Project Title: GENERAL AND REPRODUCTIVE HEALTH ASSESSMENTS OF SHORTNOSE STURGEON USING LAPAROSCOPY AND BLOOD SAMPLE ANALYSIS

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Executive summary

The Shortnose sturgeon *Acipenser brevirostrum* is one of about 27 species of sturgeon and paddlefish (Order Acipenseriformes) worldwide, all of which are either endangered, threatened or are considered vulnerable to overexploitation. Overfishing, loss of critical habitat, loss of access to spawning grounds as a result of dam construction, and pollution have contributed to declines in sturgeon populations worldwide. The high value of caviar and black market trade continues to put extensive pressure on remaining wild stocks. Effective conservation and management efforts for these species are dependent upon identification and mitigation of potential threats to the populations, such as removing dams or implementing effective fish passage measures, restoring critical habitat and protecting stocks from overfishing. Chronic affects from exposure to pollutants are less obvious, but may be an important limiting factor in the health and reproductive capacity in some populations. Exposure to a wide variety of compounds (*aka* endocrine disrupting compounds, or EDCs), often present in agricultural, municipal and industrial effluents, can disrupt a number of physiological functions in fish and other aquatic animals. Exposure to EDCs has been linked to a variety of chronic reproductive health issues in fish, notably intersex, but also decreased immune function. Reproductive and health assessments of sturgeon and other fishes depend on the ability to collect detailed physiological information. However, the imperiled status of sturgeon requires the use of safe and effective non-lethal techniques to collect data. To that end, surgical techniques (laparoscopy) were developed to assess the reproductive status of Delaware River fish and to examine internal organs for parasites or other signs of disease. Clinical pathology (a suite of hematologic and plasma chemistry analytes) was also employed to assess the health of Delaware River fish, and fish that were impeded from completing spawning runs on the Cooper River. Intersex and altered hormone profiles were identified in 11.6% of fish from Delaware River, likely as a result of EDC contamination. Physiological stress, indicated by a classic stress leukogram, was evident in fish barred from upstream spawning migration in the Cooper River as a result of inadequate fish passage measures to by-pass Pinopolis dam. Shortnose sturgeon aggregate in the tailrace below the dam and are subjected to a variety of stressors related to crowding, unsuitable habitat and inability to migrate and spawn. The physiological and morphological information collected in this study was useful to detect cryptic, sublethal effects of pollution or degraded habitat, with minimal impact to the fish examined. Hematologic and biochemical reference intervals can be important tools to diagnose disease and monitor the effects of environmental change, anthropogenic impacts or the effectiveness of management actions.

The objectives of this project:

1. Determine sex and reproductive status of fish (laparoscopy) collected from the Delaware and Cooper Rivers.
2. Evaluate the health of individuals in these populations using hematologic and biochemical analyses (blood sample analysis), as well as internal and external visual assessment, and compare these results to established reference intervals.
3. Determine circulating levels (plasma) of testosterone, estrogen, and vitellogenin (vtg) in sexed fish from these populations and compare to levels obtained from hatchery-reared fish.
4. Evaluate water samples collected from fish capture sites for presence of estrogenic compounds by use of ELISA assay.

Results reported here include all data collected from shortnose sturgeon (Delaware and Cooper Rivers) since the inception of this project, which predates the period of this present grant. Funding for this project was originally received by the National Fish and Wildlife Foundation (grant #s 2003-0206-002, 2004-0012-002, 2006-0087-001). Sampling for shortnose sturgeon began in November, 2006, in the Delaware River, and in February, 2005 in the Cooper River, after amendments to scientific research permits were completed. Permit # 1486 (originally) authorized capture and sampling of up to 24 fish/year from the Delaware, and permit # 1505 (originally) authorized data collection of up to 12 fish/year from the Cooper River. Results of this project are presented in the following chapters:

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In addition to these results, copies of the following publications are submitted, which detail use of laparoscopic techniques and physiological effects of the anesthetic protocol used in this project.

Matsche, M.A. *et al.* (2011) Use of laparoscopy to determine sex and reproductive status of shortnose sturgeon (*Acipenser brevirostrum*) and Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*). *Journal of Applied Ichthyology,* 27**,** 627-636. (Not funded by this grant, but funded by previous grants from NFWF. Juvenile Atlantic sturgeon were used as a surrogate for shortnose sturgeon for post-surgical observations).

Matsche, M.A. (2011) Evaluation of tricaine methanesulfonate (MS-222) as a surgical anesthetic for Atlantic sturgeon *Acipenser oxyrinchus oxyrinchus*. *Journal of Applied Ichthyology,* 27**,** 600-610. (Not funded by this grant. Juvenile Atlantic sturgeon were used as a surrogate for shortnose sturgeon).

The following manuscript is in preparation for publication in a peer reviewed journal. This work was not funded by this grant but will provide details on the physiological effects of surgical procedures used in this study. Juvenile Atlantic sturgeon were used as a surrogate for shortnose sturgeon.

Matsche, M.A. (in preparation) Clinical pathology and recovery of juvenile Atlantic sturgeon following laparoscopic surgery.

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Chapter 1: Reproductive demographics, intersex, and altered hormone levels in shortnose sturgeon from the Delaware River, USA

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Summary

The reproductive structure of the shortnose sturgeon population in the Delaware River was studied by examination of fish captured by gillnet from an aggregation site near Bordentown, New Jersey. Morphology of the gonads was assessed by laparoscopic observation and biopsy, and testosterone and estradiol levels were determined by ELISA assay of plasma. A total of 68 fish were examined in May-June, and 61 additional fish were examined in November during 2006-2011. Six stages of reproductive development were identified in females and 5 stages were identified in males, encompassing differentiation through maturity in both sexes. Fish captured in the spring were predominantly immature with a higher proportion of females (1:1.2 M:F sex ratio), while mature males predominated in the fall (5.7:1 M:F), indicating that the Bordentown area serves as an overwintering/pre-spawn aggregation site. Three distinct forms of intersex were noted in a total of 11.6% of fish examined: ovo-testis, consisting of scattered spermatic cysts in predominantly immature ovary; testis-ova, consisting of ovarian lamellae projecting from a predominantly immature testes; and zonal distribution, consisting of multiple, nearly-homogenous pockets of either testicular or ovarian tissues along the gonad. The hormone profile in fish with ovo-testis was similar to that of immature males, while the hormone profile in fish with testis-ova was similar to that of immature females. Zonal intersexuality may be an advanced form of male-to-female sex reversal. The diversity in gonad morphology and hormone profiles in intersexual fish may indicate that multiple causes of reproductive alterations such as exposure to contaminants (e.g. endocrine disrupting compounds) and habitat degradation (e.g. hypoxia) are involved. The relatively high prevalence of intersex in this population raises concerns regarding potential reproductive effects and long-term impacts on the population in the Delaware River.

Introduction

Shortnose sturgeon (*A. brevirostrum*) are native to major tributaries along the Atlantic coast of North America (Kynard 1997). Over-fishing following the establishment of the United States caviar industry in 1870 and additional anthropogenic factors (e.g. declining water quality, dams, and loss of spawning habitat) contributed to dramatic declines in their populations (Kynard 1997). The shortnose sturgeon was placed on the federal Endangered Species List in 1967 and remains endangered throughout its range since enactment of the Endangered Species Act in 1973 (NMFS 1998).

Shortnose sturgeon occur throughout the Delaware River estuary and rarely the nearshore ocean (Brundage & Meadows 1982). The abundance of adults is greatest in the upper tidal Delaware River from Trenton, NJ, to Philadelphia, PA (Hastings *et al.* 1987). Spawning occurs primarily in the lower non-tidal river during April (Brundage 1986; ERC 2008). After spawning, adult shortnose sturgeon disperse and spend the summer and early fall foraging throughout the tidal river, with some fish moving into Delaware Bay (Brundage and Meadows 1982; O'Herron *et al.* 1993; ERC 2006a). Most adults overwinter in dense aggregations in the Roebling, Bordentown, or Trenton reaches of the upper tidal river from December through March (O' Herron *et al.* 1993), although some may overwinter in the lower tidal river (ERC 2006a). The adult shortnose sturgeon population in the Delaware River appears to be stable at around 12,000 individuals, based on mark-recapture data collected during 1981-1984 (Hastings *et al.* 1987) and 1999-2003 (ERC 2006b).

Juvenile shortnose sturgeon occur throughout the tidal portion of the Delaware River (Hastings 1983; Brundage & O'Herron 2009). The seaward distribution of juveniles in the lower tidal river may be limited in the summer by the progressively increasing salinity levels below Wilmington, DE and the low dissolved oxygen concentrations in the Philadelphia area (Brundage & O'Herron 2009). Juvenile shortnose sturgeon and Atlantic sturgeon co-occur in the same region of the tidal Delaware River and do not segregate on the basis of salinity, as has been described in other rivers (Brundage & O'Herron 2009). Juvenile shortnose sturgeon appear to overwinter in a dispersed fashion rather than in the dense aggregations typical of the adults (Brundage & O'Herron 2009).

While the population size and dynamics have been studied in the Delaware River, the general and reproductive health condition of the fish is unknown. There has been increased awareness in recent years concerning the sublethal effects of pollution and degraded habitat on fish health (Hartwell *et al.* 2001, Webb & Doroshov 2011). Contamination of aquatic habitats may put fish at increased risk of infection, immune-related disorders, neoplasms,

and impaired reproductive function (Hinton 1989, McMaster 2001, Milla *et al.* 2011). Many types of contaminants have been labeled as endocrine disrupting compounds (or EDCs) as they can mimic endogenous hormones, alter the natural pattern of hormone synthesis or metabolism or modify hormone receptor levels (Scholz & Klüver 2009). Exposure of fish to EDCs can result in delayed onset of maturity, intersex (i.e. co-development of male and female characteristics), malformed gonads, decreased gonadal-somatic index, and skewed sex ratios (Scholz & Klüver 2009). Impaired reproduction or altered sex ratio could limit recovery of the shortnose sturgeon population in the Delaware River. However, information on the reproductive condition of shortnose sturgeon in the Delaware River, including potential effects of EDCs, is lacking. Therefore the goal of this study was to document the reproductive structure of the shortnose sturgeon population in the Delaware River and to examine gonad development and plasma hormone levels for evidence of endocrine disruption.

Materials and Methods

Shortnose sturgeon used in this study were captured from the upper tidal Delaware River, near Bordentown, NJ (see Fig. 1 in Matsche *et al.* 2011) using anchored bottom-set gill nets (100 m long by 1.8 m deep, 12.7 or 15.2 cm stretched monofilament mesh). Sampling was conducted in the spring during May 22nd – June 3rd, and in the fall during November 2nd – November 9th, 2006-2011. Upon capture, fish were examined, measured, weighed, and tagged (passive integrated transponder tag and external Floy® anchor tag). Fish were anesthetized in tricaine methanesulfonate (MS-222), examined internally, and the gonad biopsied according to the procedures of Matsche *et al.* (2011). Briefly, a 5-mm laparoscope and biopsy forceps were inserted into the coelom of anesthetized fish through a pair of cannulae installed in the ventral body wall. Gonads were examined for gender and maturity and a single gonadal biopsy was collected and placed into formalin. Aspects of intestine, spleen, stomach, peritoneum, liver and pancreas that were readily observable laparoscopically were briefly examined for lesions in most fish. Organs such as intestine, spleen, and liver were moved to enhance observations in some fish by using a closed biopsy forceps or the distal tip of the laparoscope. The incisions were closed with size 0 or 2-0 PDS II plus sutures (Ethicon Inc., Somerville, NJ) after the instruments were removed. The fish were then recovered in fresh water and released.

Formalin-fixed biopsies were processed for routine paraffin infiltration, sectioned at 7 µm, and stained with Mayer's hematoxylin and eosin (Presnell & Schreibman 1997). The reproductive stage of testicular tissue was

determined by the relative abundance of cysts with spermatogonia, spermatocytes, spermatids, and spermatozoa. The reproductive stage of ovarian tissue was determined by oocyte size; position and staining characteristics of the germinal vesicle; presence and thickness of the vitelline envelope and relative amount of lipid vacuoles, yolk platelets and melanin. Classification of ovarian tissue was based on the most advanced oocytes present. The diameters of at least 20 oocytes per female were measured using an image analysis system consisting of a BX50 microscope, DP25 digital camera, and DP2-BSW software, v 2.1 (Olympus America, Inc., Center Valley, PA).

While under anesthesia, a 3-5 ml blood sample was collected from the caudal vein of each fish and transferred to blood collection tubes containing lithium heparin and plasma separator gel (600-µl Microtainer® and 3-ml Vacutainer®, BD, Franklin Lakes, NJ). Blood in 3-ml tubes was centrifuged for 10 min at 10,000 xg. Plasma was decanted into cryovials, placed on dry ice for transport and stored at -80ºC for later hormone analysis. Blood collected in 600-µl tubes was used for hematology and plasma chemistry analysis, the results of which will be reported separately (Chapter 2).

Plasma testosterone and estradiol concentrations were measured using EIA ELISA kits (models 582701 and 582251, Cayman Chemicals, Ann Arbor, MI), according to the manufacturer's instructions. All samples were extracted in diethyl ether, the cold spike method was used to determine extraction efficiency and the samples were analyzed with a uQuant spectrophotometer (Biotek Instruments, Inc., Winooski, Vermont, USA) at a wavelength of 410 nm.

Fulton's condition factor (K) was determined for each fish according to Ricker (1975). Weight, total length (TL), K and hormone concentration were tested for normal distribution (Shapiro-Wilk) and homogeneity of variance (Bartlett). Pearson correlation coefficients were used to compare the relative effects of year, season, gender and maturity on K, testosterone and estradiol. Multiple one-way ANOVA followed by sequential Bonferroni post-hoc tests (Rice 1989) were used to compare weight, TL, K, estradiol and testosterone among each combination of gender (including intersex) and reproductive stage. The statistical analyses were performed using SAS Enterprise Guide 4.1 (Davis 2007).

Results

A total of 129 shortnose sturgeon were captured from the Delaware River during 2006-2011 and examined using laparoscopy. Five stages of testicular development were identified among 73 males and six stages of ovarian

development were identified among 41 females, encompassing differentiation through maturation stages. Post-spawn females or males were not observed in this study. Classification of testicular development is summarized in Table 1 and ovarian development is summarized in Table 2. See Matsche *et al.* 2011 for appearance of gonads when viewed laparoscopically. In addition, 15 of the 129 fish (11.6%) had altered gonadal development consisting of mixed testicular and ovarian tissue to varying degrees, indicating intersex. Three patterns of altered development were identified among these fish (see below). No lesions or parasites were evident in other internal organs examined.

Testicular development

Stage 1 - Differentiation. (n=3). Testes were bound by an outer tunica albuginea and consisted primarily of adipose tissue with a narrow band of germinal tissue along the mesorchium. Spermatogonia were not encysted and individual or clusters of adipocytes were commonly scattered among gonial tissue (Fig. 1a). Gonads appeared as a thin, yellow ribbon of tissue.

Stage 2 - Proliferation. (n=20). The germinal layer was thicker than in stage-1 testes, but adipose tissue still predominated. Spermatogonia were encysted and small amounts of residual spermatozoa were occasionally present in some fish. Adipocytes were often found scattered among germinal tissue (Fig. 1b). Testes were predominantly yellow with fat, but with a narrow ribbon of white germinal tissue parallel and close to the mesorchium.

Stage 3 – Early-spermatogenesis. (n=16). The majority of testicular cysts contained spermatogonia or spermatocytes, while <10% of the cysts contained small clusters of spermatids. The Layer of adipose tissue was reduced or absent, but adipocytes were commonly found scattered among the germinal tissue (Fig. 1c). Appearance of testes at this stage varied. Some testes were all white, while others were mostly yellow, particularly on the medial margin, with thin streaks of yellow fat over the surface.

Stage 4 – Mid-spermatogenesis. (n=13). A distinct adipose tissue layer was absent but adipocytes were found scattered among the germinal tissue. The majority of testicular cysts contained spermatocytes or spermatids,

while < 10% of cysts contained spermatogonia or spermatozoa (Fig. 1d). Testes were predominantly white in color and were moderately large in size.

Stage 5 – Maturation. (n=21). Testicular cysts contained differentiated spermatazoa and adipose tissue was virtually absent (Fig. 1e). Mature testes filled much of the coelom and appeared white with no visible fat on the surface.

Ovarian development

Stage 1 – Differentiation. (n=1). The ovaries were bound within an outer tunica albuginea and consisted primarily of adipose tissue with narrow band of germinal tissue along the mesovrium organized in a lamellar arrangement (Fig. 2a). Ovarian lamellae contained an inner layer of oogonia and an outer columnar epithelium (Fig. 2a). Individual or small clusters of oocytes with heterochromatic nuclei were scattered among the oogonia (Fig. 2a). Gonads appeared as a thin ribbon of yellow fat. Ovarian lamellae were not readily apparent by observation alone, but were evident by palpation of the gonad surface with blunt forceps.

Stage 2 – Endogenous growth of oocytes. (n=15). Ovarian lamellae consisted primarily of adipose tissue, with stage 2 oocytes, lesser numbers of stage 1 oocytes and small clusters of oogonia scattered along the tunica albuginea (Fig. 2b). Perinucleoli, granules, and occasional lampbrush chromosomes were present in the germinal vesicle of stage 2 oocytes. The ooplasm was dark basophilic with a ring of lipid vacuoles circumferential to the germinal vesicle, and oocytes contained an outer layer of follicle cells (Fig. 2b). The germinal surface of stage-2 ovaries appeared pink with a granular texture and ovarian lamellae were readily observed.

Stage 3 – Early-vitellogenesis. (n=8). Adipose tissue in the inner stroma of the ovarian lamellae was reduced as oocytes increased in size and number. Ooplasm of stage 3 oocytes was light-basophilic in color with a central ring of lipid vacuoles and a single-layered vitelline envelope (<5 µm thick) below the follicle cell layer (Fig. 2c). The germinal vesicle was enlarged, light-eosinophilic in color, and occasionally contained granules and lampbrush chromosomes (Fig. 2c). Variable numbers of stage-2 oocytes and a reduced amount of oogonial tissue were present. Ovaries were yellow to pink in color and dotted with small, white oocytes.

Stage 4 – Mid-vitellogenesis. (n=6). Ovarian lamellae were greatly hypertrophied with numerous, large oocytes. Stage 4 oocytes had a light-basophilic cytoplasm with a band of eosinophilic yolk platelets concentrated at the periphery and reduced numbers of yolk platelets scattered centripetally (Fig. 2d). The vitelline envelope was 5-10 µm thick with 2 faintly differentiated layers (Fig. 2d). The germinal vesicle was light-eosinophilic in color and typically contained lampbrush chromosomes and granules (Fig. 2d). Larger stage-4 oocytes had an expanded ring of yolk platelets centripetally with fewer yolk plates at the periphery and lipid vacuoles were larger in size and concentrated between the ring of yolk platelets and germinal vesicle. Diversity of ovarian development was highest in stage 4, which included numerous stage 2-3 oocytes and few oogonial nests and stage 1 oocytes. Stage-4 ovaries were moderately large in size and appeared lumpy with numerous yellow oocytes and little fat.

Stage 5 – Migratory nucleus. (n=5). Overies were greatly enlarged and consisted primarily of oocytes bound within a loose, fluid-filled stroma with few, scattered stage 1-2 oocytes and little adipose tissue (Fig. 2e). The ooplasm of stage 5 oocytes was filled with numerous eosinophilic yolk platelets and contained a thin layer of melanin pigment immediately subjacent to the vitelline envelope (Fig. 2f). The germinal vesicle was displaced towards the animal pole and lipid vacuoles were concentrated towards the vegetal pole (Fig. 2e). The vitelline envelope was 30-50 µm thick with 2 distinct layers and occasionally with a third faintly differentiated layer (Fig. 2e). Stage-5 ovaries filled much of the coelom, and appeared as a mass of large grey eggs, scattered with lesser numbers of small, white oocytes.

Stage 6 – Oocyte maturation. (n=6). Oocytes were not bound within an ovarian stroma, but occasionally, one or two stage-2 oocytes were bound to an individual stage-6 oocyte within a thin thecal envelope. The **v**itelline envelope was 100-140 µm thick and consisted of 3 distinct layers with the micropile at the animal pole (Fig. 2f). The germinal vesicle was absent, a thin layer of melanin pigment was located immediately subjacent to vitelline envelope, and lipid vacuoles were concentrated in the vegetal hemisphere (Fig. 2f). All stage-6 oocytes examined histologically were pre-ovulatory. Mature black eggs filled the coelom and were readily apparent upon incision or insertion of a cannula in these fish.

Intersex

A total of 15 fish (11.6%) exhibited altered gonadal development indicating intersex. Three categories of intersex were identified based on morphological (visual assessment using laparoscope) and histological examinations of the gonads.

Ovo-testis. (n=5, 3.9% of fish examined). Fish were categorized as female upon visual examination of the gonads using the laparoscope. Ovaries in these fish had a granular, pink lamellate surface, or were nodular with white to yellow oocytes, and with a variable amount of ovarian fat. Microscopic examination revealed that the gonads consisted primarily of immature or developing ovarian tissue, but with few spermatic cyst-like structures scattered along the tunica albuginea (Fig. 3a). The cyst-like structures often contained a central lumen surrounded by or containing spermatogonia, sertoli cells or unidentified cells (Fig. 3b). Occasionally, spermatids or mature spermatozoa were present in the lumen of the cyst-like structures (Fig. 3c). In four of the five fish, ovarian tissue in the intersexual gonads resembled typical endogenous growth phase (Fig. 3a), while the fifth fish had predominantly mid-vitellogenic tissues.

Testis-ova. (n=6, 4.7% of fish examined). Testis-ova appeared as one or few small clusters of white or yellow oocytes in the surface of immature testis (Fig. 4a). Microscopic examination revealed that the clusters consisted of ovarian lamellae, containing primarily stage 2 oocytes and lesser numbers of stage 1 oocytes and oogonia, arising from the tunica albuginea of the testis (Fig 5a). In all six fish examined, the testicular portion of the intersexual gonad contained encysted spermatogonia (stage 2 males, Fig. 5a,b). However, spermatic cysts in close proximity to the testis-ova occasionally exhibited atypical morphology, including a general loss cyst organization, multifocal hyperplasia of the interstitial cells and reduced number of spermatogonia (Fig. 5c). Cyst-like structures were also commonly found intermingled within the ovarian lamellae, containing an unorganized mixture of spermatids, spermatocytes and spermatogonia (Fig. 5d), or less frequently, with spermatids or spermatozoa (Fig. 5e).

Zonal. (n=4, 3% of fish examined). Gonads consisted of distinct bands or regions of either apparent testicular or ovarian tissue (Fig. 4b). Relative amount of testicular and ovarian tissues appeared to be similar in all fish with

zonal intersexuality. Subjective evaluation indicated that more of the testicular zones were located laterally (adjacent to the mesorchium or mesovarium) than ovarian zones. Microscopic examination revealed that the testicular zones were mature (majority of cysts contained spermatozoa) and that the ovarian zones were maturing (contained either stage-4 or stage-5 oocytes, Fig. 6a). In 3 of 4 fish, both gonads contained intersexual zones. In the 4^{th} fish, the right gonad was intersexual, while the left gonad appeared entirely testicular and contained only mature spermatozoa in the biopsy. Intermingling of testicular and ovarian tissues was uncommon and limited to the margins near the juncture of testicular and ovarian zones (Fig. 6b,c). Mixed ovarian and testicular tissue was not evident in biopsies collected from the center of either testicular or ovarian zones.

Season and fish size demographics

A significantly higher proportion of females were captured in the spring (1:1.2 (M:F) sex ratio), while a significantly higher proportion of males were captured in the fall (5.7:1 sex ratio) (Table 3). Fish captured in the spring were mostly immature, while mature males predominated in the fall (Fig. 7).

Stage 5 and 6 females had significantly higher mean TL than males and stage 1-4 females (corrected total d.f. = 11, $F = 5.80$, $P < 0.0001$) and were generally higher in mean weight (corrected total d.f. = 11, $F = 5.13$, $P < 0.0001$) than less mature fish (Fig. 8). Condition factor differed by gender, was higher in the fall and increased with reproductive maturity (Table 4, Fig. 9).

Steroid hormones

Average extraction efficiencies were 82.2% for testosterone and 89.6% for estradiol. The reported detection limits of the ELISA kits at %B/Bo of 80% were 6 pg ml^{-1} for testosterone and 19 pg ml^{-1} for estradiol. The reported cross-reactivity of the testosterone antibodies is 27.4% for 5α-dihydro testosterone, 18.9% for 5β-dihydro testosterone and <5% for other similar steroids. The cross-reactivity of the estradiol antibodies is 14% for estradiol-3-glucuronide, 12% for estrone and ≤1% for other similar steroids. The intra-assay variation of the ELISA kits was 8.9% for testosterone and 4.5% for estradiol. The inter-assay variation was 10.2% for testosterone and 7.6% for estradiol.

Testosterone and estradiol concentrations were higher in the fall, were significantly affected by gender and generally increased with maturity (Table 5). Testosterone was highest in mature males (corrected total d.f. = 13, $F = 100.15$, $P < 0.0001$) (Fig. 10). Overall, estradiol was highest in fish with zonal intersex gonads (Fig. 10). Within

females, estradiol increased with development to stages 4-5 and then declined in stage 6 (corrected total d.f. = 13, F = 50.24, P < 0.0001) (Fig. 10). For all but stage 1 fish, T:E2 ratios were significantly higher for males than females and intersex fish (corrected total d.f. = 13, F = 18.51, P < 0.0001) (Fig. 11). T:E2 ratios did not differ by reproductive stage in females or between females and fish with intersex condition (Fig. 11).

Discussion

Laparoscopic techniques provides a useful, minimally invasive method of assessing reproductive condition in sturgeon and other species where lethal sampling is to be avoided (Hernandez-Divers *et al* 2004, Swenson *et al.* 2007, Matsche *et al.* 2011). Only actively spawning sturgeon with freely flowing gametes can be reliably sexed by external observations alone (Bruch *et al.* 2001). Gonadal tissue must be examined in all other fish, either by direct observation (i.e. surgical or necropsy) or by biopsy, although biochemical markers may be useful in some species (Web & Doroshov 2011). In this study, fish were examined using laparoscopy in as little as 2 min. While the anesthetic protocol (MS222) used in this study was safe and effective (Matsche 2011), anesthesia increased the total handling time of fish by as much as 20 min. Laparoscopy can be effective in assessing a much larger sample size of fish if a rapid anesthetic protocol is used (Hurvitz *et al.* 2007). Alternate anesthetic techniques for field surgical procedures, including laparoscopy, need to be evaluated for use with sturgeon (Henyey *et al.* 2002).

In this study, a male to female sex ratio of 1:1.2 was observed among shortnose sturgeon collected in the spring. This ratio was within the range described for the general population (i.e., not on the spawning grounds) of shortnose sturgeon in other estuaries. Dadswell (1979) reported that the male:female ratio of shortnose sturgeon in the St. John River, Canada ranged from 1:1 to 1:2, with the proportion of females increasing with age. Kynard *et al.* (unpublished data) reported a sex ratio of adult shortnose sturgeon in the Connecticut River of near 1:1, while Greeley (1937) noted a male:female ratio of 1:1.42 for shortnose sturgeon in the Hudson River.

Present-study collections in fall were dominated by males, which occurred at a ratio 5.7:1. These collections sampled individuals from the overwintering/pre-spawn aggregation in the upper tidal river (O'Herron & Able, 1987) and the sex ratio observed may reflect that expected to occur on the spawning grounds the next spring. A preponderance of male shortnose sturgeon on the spawning grounds has been reported for the Delaware River (29.6:1, ERC 2006a), the Hudson River (2.5:1, Pekovitch 1979), and the Connecticut River (9.9:1 to 11.2:1, Kieffer & Kynard in press). The high proportion of males on the spawning grounds is related to the likelihood of many

males, but few females, spawning annually. Spawning intervals exceeding 10 years have been reported for females in some shortnose sturgeon populations (Dadswell *et al.* 1984).

Acoustic telemetry studies in the Delaware River suggest that shortnose sturgeon, particularly females that are not going to spawn the next spring, may not overwinter in the upper tidal river aggregation (ERC 2006b, ERC & AOI 2010). This shifts the sex ratio of fish in this aggregation in favor of males. The results of the present study demonstrate that differential use of various regions of the estuary by males and females may skew the perceived sex ratio of the general shortnose sturgeon population because it is spatially and temporally variable.

It is interesting that no post-spawn shortnose sturgeon were observed in this study. It is likely that post-spawn fish had already moved through the area by the time sampling was conducted. Spring sampling in this study was performed approximately 1 to 1.5 months after the spawning period. Acoustic telemetry has demonstrated that post-spawn shortnose sturgeon in the Delaware River rapidly migrate variable distances seaward immediately after spawning (O'Herron *et al.* 1993, ERC 2006b, ERC & AOI 2010). Some fish remain in the upper tidal river, but typically below the area sampled in the present study, while others move to the lower tidal river or upper Delaware Bay. Most of the longer distance migrants will move back to the upper tidal river by fall or early winter, although some overwinter in the lower tidal river (ERC 2006b, ERC & AOI 2010).

Laparoscopy was useful in detecting intersex in 11.6% of the shortnose sturgeon population in this study, either by observation of intersex tissues through the laparoscope or by subsequent microscopic evaluation of biopsies. Intersex in gonochoristic fish such as sturgeon is expected to be rare. Indeed, frequency of intersex encountered in wild populations of sturgeon is typically low; <0.01% in white sturgeon from San Francisco Bay (Chapman *et al.* 1996), 2% in shovelnose sturgeon from the Mississippi River (Colombo *et al.* 2004, 2007), 1% in Atlantic sturgeon from Hudson River (Van Eenennaam & Doroshov 1998) and 3% in shovelnose sturgeon from Mississippi and Missouri Rivers (Carlson *et al.* 1985). Low rates of intersex may occur naturally in sturgeon or may indicate mild effects of pollution or other causes. In shortnose sturgeon, intersex has previously been documented in a single cultured specimen (Henne *et al.* 2006), and from a single fish from the Hudson River (Atz & Smith 1976). Increased prevalence of intersex in fish populations is often associated with environmental contamination including presence of EDCs (Harshbarger *et al.* 2000, Mills & Chichester 2005).

The prevalence of intersex in shortnose sturgeon in the Delaware River reported here may underestimate actual rates based on limitations of the diagnostic techniques used in this study. While excellent image quality is achieved

with a rod-lens laparoscope, observation of the gonads is limited compared to necropsy, and germinal tissue is often obscured by extensive gonadal fat in immature fish (Matsche *et al.* 2011*)*. In this study, volume of tissue collected in biopsies was small and sample location was biased towards the periphery of the organ in fish with large, mature gonads. Intersexual tissues (ovo-testis or testis-ova) do not appear to be evenly distributed in sturgeon gonads, are not always apparent by gross examination and could be missed if located deeper within the stroma of the organ. Consequently, the probability of detecting mild forms of intersex by morphologic assessment of gonads with a laparoscope, or by examination of few, small biopsies, is likely reduced compared to necropsy (Blazer *et al.* 2007).

The morphological features of intersex can vary considerably in sturgeon. Intersex may indicate a "feminization" process, in which ovarian tissues develop in male testes (e.g. testis-ova), or "masculinization", in which testicular tissues develop within female ovaries (e.g. ovo-testis). Severity of intersex can vary from mild, consisting of few scattered oocytes in a testis (Van Eenennaam & Doroshov 1998) or few scattered spermatic cysts in an ovary (this study), to severe forms with extensive co-development of ovarian and testicular tissues (Chirkina 1957, Henne *et al.* 2006, Jackson *et al* 2006). Testicular and ovarian tissues can be disbursed throughout an intersex gonad with extensive intermingling (Atz & Smith 1976), but more often in sturgeon, gonads exhibit a bilaterally asymmetric distribution (Chirkina 1957, Henne *et al.* 2006, Jackson *et al.* 2007). In some fish, only a single gonad may be affected (Ziemiankowski 1954). Ovarian and testicular tissues can be separated cranio-caudally within an intersex gonad (Atz & Smith 1976, Henne *et al.* 2006) or develop within multiple pockets scattered along the length of the organ (Colombo *et al.* 2007, Jackson *et al.* 2006). The term "hermaphrodite" describes an evolved reproductive strategy and is often used incorrectly to describe pathological occurrence of intersex in fish (Sadovy and Shapiro 1987).

To our knowledge, this is the first report of ovo-testis in sturgeon. In fish, ovo-testis may result from exposure to androgenic EDCs, from a general disruption in hormone levels during sexual differentiation or from exposure to hypoxia (Dietrich & Krieger 2009, Thomas and Rahman 2011). Masculinizing effects such as ovo-testis are less commonly reported in fish than feminizing effects, perhaps because androgenic compounds are less prevalent in the environment or are converted to estrogens (e.g. aromatization) following uptake in fish (Dietrich & Krieger 2009). Few, small areas of male germ cells (spermatocytes to spermatozoa) were found along the periphery of the ovaries, similar to the distribution of ovo-testis in shortnose sturgeon reported here, in female Atlantic croaker, *Micropogonias undulatus*, collected from hypoxic regions in the northern Gulf of Mexico (Thomas & Rahman

2011). A potential mechanism for ovo-testis and other masculinizing effects in female fish is suppression of aromatase activity. Aromatase converts androgens to estrogens in natural synthetic pathways and is essential for normal ovarian development in fish (Guigen *et al.* 1999). Hypoxia and certain chemical contaminants can suppress aromatase activity in fish leading to altered hormone levels, phenotypic male characteristics in females, including intersex (Piferrer *et al.* 1994, Nakamura *et al.* 2003, Guigen *et al.* 2010), or sex ratios skewed towards males (Shang *et al.* 2006). Furthermore, fish with suppressed aromatase activity may be more susceptible to other environmental contaminants. Levels of androgenic EDCs are unknown and hypoxia is not common in the Delaware River (Sharp 2010). However, summertime hypoxia was extensive as recently as the late 1980s (Sharp 2010), well within the lifespan of this long-lived species and may continue to form on a limited basis.

Testis-ova in sturgeon may consist of ovarian lamellae extending from the tunica albuginea, but can also occur as pockets of ovarian tissues embedded within the testicular stroma (Van Eenennaam & Doroshov 1998, Harshbarger *et al.* 2000). In this study, the small foci of testis-ova in the surface of the gonad also contained mature testicular tissue intermingled among the oocytes, within an otherwise immature testis. In contrast, ovarian lamellae projecting from the tunica albuginea of intersexual testis in shovelnose sturgeon did not contain testicular tissue (Harshbarger *et al.* 2000). While the gender of fish with mild to moderate forms of intersex may be assumed based on the relative proportion of tissue types within the gonads, fish with zonal intersexual gonads had roughly equal proportions of ovarian and testicular tissues, and therefore the innate gender was not obvious. It may be hypothesized however, that zonal intersexuality observed in shortnose sturgeon in this study, is an advanced form of male sex reversal. One fish in this study had one apparently normal testis and one intersexual gonad, and the only moderate form of intersexuality observed in this population was testis-ova.

Trends in testosterone and estradiol with gonadal development stages in males and females observed here is similar to the hormone profiles reported for other sturgeon species (Amiri *et al.* 1996a,b, Van Eenennaam *et al.* 1996, Barannikova *et al.* 2004). In females, estradiol concentrations tend to increase with vitellogenesis, but then decline during the final stages of maturation (Amiri *et al.* 1996a, Van Eenennaam *et al.* 1996, Barannikova *et al.* 2004, Semenkova *et al.* 2002). Estradiol is positively correlated with development stage of ovaries, as estradiol mediates oocyte growth and vitellogenesis in fish (Fostier *et al.* 1983). Final oocyte maturation is induced following a steroidogenic shift from estradiol to maturation-inducing hormone (Nagahama *et al.* 1993). Testosterone also increases with maturity in females (Amiri *et al.* 1996b, Barannikova *et al.* 2004) as estradiol is synthesized by

aromatization of testosterone within granulosa cells (Fostier *et al.* 1983). In males, testosterone is generally correlated with maturity but may decline following spermatogenesis (Amiri *et al.* 1996a, Barannikova *et al.* 2004) as a result of decreased androgen conversion and increased progestin synthesis just prior to spermiation (Kime 1993). Estradiol synthesis is usually low in male sturgeon and may not fluctuate to a great extent during development (Webb *et al.* 2002, Wildhaber *et al.* 2007). The role of estradiol in males is unclear but it has been linked to spermatogonial proliferation in fish (Dietrich & Krieger 2009). Range in values of estradiol and testosterone reported in fish can vary considerable among species, between wild and captive fish and as a result of differences in diet and analytical techniques (Kime 1993, Barannikova *et al.* 2004).

The observed steroid profiles for intersex fish offer an interesting contrast to the morphology of the gonads. Hormone levels in fish with ovo-testis were closer to levels found in immature males, even though the predominant tissue type was immature ovary. In contrast, fish with testis-ova in predominantly immature testes had elevated levels of estradiol compared to immature males. Finally, intersexual fish with approximately equal amounts of mature ovary and testis (zonal distribution) had significantly lower testosterone than in mature males, and significantly higher estradiol than in mature females. Hormone profiles were also examined in cultured Russian sturgeon with intersex gonads (Jackson *et al.* 2006). The gonads of intersexual Russian sturgeon were comprised mostly of immature ovary with scattered pockets of mature testicular tissue (Jackson *et al.* 2006). The estradiol and 11-ketotestosterone levels in those intersexual fish were closer to that of males, possibly because the testicular tissues were mature while the ovarian tissues were immature (Jackson *et al.* 2006).

The Delaware River estuary is highly industrialized and developed and, as such, has been subject to historical and current input of toxic chemicals from numerous point and non-point sources. These chemicals include heavy metals and a variety of organic compounds, including polyaromatic hydrocarbons (PAHs), PCBs, pesticides, dioxins, and furans (Sutton *et al.* 1996, Costa & Sauer 2004). Contaminant concentrations in the water column have generally declined in recent decades (Kreeger *et al.* 2006). However, many compounds, particularly chlorinated organics, tend to partition to the sediment, and concentrations remain above sediment quality criteria in many areas (Hartwell *et al.* 2001). Sediment concentrations of PAHs, PCBs, and chlorinated pesticides are generally elevated in the upper estuary, peak in the Philadelphia-Camden area, and show a declining trend seaward through the lower estuary and Bay (Costa & Sauer 2004). Bioaccumulation of toxic chemicals by fish and shellfish in the Delaware River estuary has been well documented (Gottholm *et al.* 1994, Kennish & Ruppel 1996a,b, Ashley *et al.* 2009) and

fish consumption advisories have been issued by the states of Delaware, New Jersey, and Pennsylvania because of mercury, PCBs, chlorinated pesticides, and dioxin. Sturgeon may be particularly vulnerable to sediment-associated contaminants because of their slow maturation, longevity, and the benthic feeding strategy.

ERC (2002) analyzed muscle, liver, and gonad tissue from two shortnose sturgeon (one male, one female) from the Delaware River. Sixteen metals, two semivolatile compounds, three organochlorine pesticides, and one PCB Aroclor (Aroclor 1260), as well as polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) were detected in one or more of the tissue samples. The TCDD total toxicity equivalent concentration (TEC) (see Walker & Peterson 1991) of PCDDs and PCDFs measured in ovary tissue was above the concentration indicated in the literature as causing early life stage mortality in salmonids (Cook *et al.* 1997). Moreover, concentrations of aluminum, cadmium, copper, PCBs and dichlorodiphenyldichloroethylene (DDE) in some samples were above adverse effect concentrations reported for other fish species in the literature. Monosson (1997) reported that the adverse effects of PCDDs/TCDFs, PCBs, and DDE may be exacerbated when they occur in combination.

Of the chemicals detected by ERC (2002), PCDDs/TCDFs, DDE, PCBs, and cadmium have been identified as endocrine disrupting chemicals (EDCs). EDCs are exogenous substances that cause "adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function" (Leung *et al.*, 2002). EDCs have been linked to reproductive and developmental disorders in many species, and have been implicated as a causative factor in the decline of some wildlife populations, including those of freshwater and marine fish (Monosson, 1997). In addition to intersex and altered steroid levels, EDCs have been documented to cause decreased testicular growth, reduced fecundity and egg viability, increased early life stage mortality, anatomical defects in larvae, delayed or precocious puberty and decreased immunocompetence and increased susceptibility to disease during gametogenesis (Leung *et al.* 2002, Monosson 1997, Milla *et al.* 2011). Decreased reproductive success is of concern in any population, but is of particular significance in an endangered species such as shortnose sturgeon, that matures at an advanced age and may spawn intermittently.

To our knowledge, there are no studies in the literature that directly correlate tissue contaminant concentrations in shortnose sturgeon with gross or histologic abnormalities or biomarker concentrations. However, Roy *et al.* (2011) demonstrated that laboratory exposure to TCDD and PCB126 at environmentally relevant concentrations induced expression of CYP1A mRNA in larvae and early juvenile shortnose sturgeon and Atlantic sturgeon. Induced expression of CYP1A has been correlated with higher level toxic effects in other fish taxa including DNA

damage, early life-stage toxicities, and several cancers (Wirgin & Theodorakis 2002). In a field study of shovelnose sturgeon from a contaminated site on the Mississippi River, intersex prevalence was 29% (2 of 7) in males with elevated tissue levels of chlordane, PCBs and DDE, while intersex was not detected in 11 males collected from an upstream reference site (Harshbarger *et al.* 2000).

Correlations between tissue concentrations of chlorinated organics, and various somatic and physiological effects have been described for other sturgeon species. Koch *et al.* (2006) reported higher concentrations of organochlorine compounds in the brain-hypothalmic-pituitary (BHP) complex in intersexual shovelnose sturgeon from the Middle Mississippi River compared with those of mature males. They also reported negative correlations between the gonadosomatic index (GSI) of mature males and organochlorine concentrations in BHP complex tissues, gonads, and fillets. Feist *et al.* (2005) found that plasma triglycerides and condition factor in white sturgeon (*Acipenser transmontanus*) from impounded areas of the Columbia River were negatively correlated with total DDT, total pesticide, and PCB concentrations in liver and gonad tissue. They also reported that plasma androgens and gonad size in males were negatively correlated with total DDT, total pesticide, and PCB concentrations.

The present finding of elevated proportion of shortnose sturgeon with intersex and altered steroid levels could have important implications for the long-term recover of this species. Trends in intersex prevalence in the Delaware River population are unknown, or if the reproductive effects observed in this study result in decreased reproductive success. The high rate of intersex (>10%) and altered hormone levels implicates chemical contamination as a potentially important limiting factor in recovery of this species. It is of some concern that the population in the Delaware River has apparently remained stable rather than increased in recent decades, in the absence of fishing pressure and with general improvements in water quality (Sharp 2010). This study provides baseline information on reproductive condition for the Delaware River population of shortnose sturgeon, which is useful for future comparisons within this population and with other population segments. Additional surveys are needed to identify contaminant levels in critical sturgeon habitat in the Delaware River and to determine if the prevalence of intersex is changing.

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Table 1
Classification of testicular maturity in shortnose sturgeon from Delaware River.

Stage		Description
1	Differentiation	Spermatogonia not encysted
2	Proliferation	Spermatogonia encysted, germinal tissue layer thickened
3	Early-spermatogenesis	Majority of cysts contain spermatogonia and spermatocytes
4	Mid-spermatogenesis	Majority of cysts contain spermatocytes and spermatids
5	Maturation	Testes enlarge with little fat and filled with spermatazoa

Table 2
Classification of ovarian maturity in shortnose sturgeon from Delaware River.

Stage		Oocyte diameter (µm) mean ± SD	Cytoplasm	Vitelline envelope	Yolk platelets	Melanin	Germinal vesicle
1	Differentiation	21 ± 9	Dark basophilic	No	No	No	Chromatin
2	Endogenous growth of oocyte	210 ± 80	Basophilic	No	No	No	Perinucleolus
3	Early-vitellogenesis	425 ± 90	Light basophilic	1 layer	No	No	Central
4	Mid-vitellogenesis	813 ± 96	Light basophilic with eosinophilic ring (bullseye)	2-3 layers	Variable	No	Central
5	Migratory nucleus	1,620 ± 150	Polarized, eosinophilic	2-3 layers	Numerous	Yes	Migratory
6	Maturation	2,240 ± 200	Polarized, eosinophilic	3 layers	Numerous	Yes	Absent

Table 3
Sex ratios of shortnose captured in the Delaware River, 2006-2011. Asterisk (*) indicates significant divergence from expected 1:1 (M:F) ratio.

Season	n	% Males	% Females	% Intersex	Fisher's Exact P
Spring	68	39.7	47.1	13.2	0.0048*
Fall	61	75.4	13.2	9.8	<0.0001*
Overall	129	56.6	31.8	11.6	<0.0001*

Table 4
Pearson correlation coefficients (PCC) for effect of year, season, gender and reproductive maturity on condition factor.

Season	n	PCC	P
Year	127	0.1324	0.0623
Season	127	0.5123	<0.0001
Sex	127	0.2779	0.0016
Maturity	127	0.5064	<0.0001

Table 5
Pearson correlation coefficients (PCC) for effect of season, gender and reproductive maturity on hormone levels. n = 126 for both hormones.

Season		Testosterone	Estradiol
Year	PCC	-0.0098	0.0992
	P	0.9120	0.2636
Season	PCC	0.6180	0.0248
	P	<0.0001	0.7801
Sex	PCC	0.1566	0.2308
	P	0.0363	0.0085
Maturity	PCC	0.6912	0.3379
	P	<0.0001	<0.0001

Figure captions

Fig. 1. Male reproductive stages identified in shortnose sturgeon from the Delaware River: (a) stage 1 differentiation, (b) stage 2 proliferation of spermatogonia, (c) stage 3 early-spermatogenesis, (d) stage 4 mid-spermatogenesis, and (e) stage 5 maturation. AC, adipocytes; SG, spermatogonia; CS, cyst; SC, spermatocytes; ST, spermatids; SZ, spermatozoa. Scale bar = 100 µm. Inset scale bar = 25 µm

Fig. 2. Female reproductive stages identified in shortnose sturgeon from the Delaware River: (a) stage 1 differentiation, (b) stage 2 endogenous growth of oocytes, (c) stage 3 early vitellogenesis, (d) stage 4 mid vitellogenesis, (e) stage 5 migratory nucleus, and (f) stage 6 maturation. AC, adipocytes; OL, ovarian lamellae; OC, oocytes $_{(\text{stage 1-6})}$; GN, oogonia; GV, germinal vesicle; NL, nucleoli; FC, follicle cells; VE, vitelline envelope; TC, thecal cells; YP, yolk platelets; ME, melanin; AP, animal pole; VP, vegetal pole; ZI, zona interna; ZE, zona externa; GC, gel coat; MP micropile. Scale bar = 100 µm (a and b), 200 µm (c and d), 500 µm (e and f). Inset scale bar = 20 µm

Fig. 3. Ovo-testis in shortnose sturgeon from Delaware River: spermatic cyst-like structures (arrow heads) were typically subjacent to the tunica albuginea (a) and were distinguished from oogonial nests (b) by the presence of a central lumen and spermatogonia (c). Mature spermatozoa were infrequently found within the spermatic cyst-like structures (d). OC, oocytes $_{(\text{stage 1-6})}$; GN, oogonia; SG, spermatogonia; SZ, spermatozoa. Scale bar = 200 µm (a), 60 µm (b), 30 µm (c), 100 µm (d)

Fig. 4. Laparoscopic view of intersex condition in shortnose sturgeon: (a) testis-ova (arrowhead) in immature testis and (b) zonal intersexuality.

Fig. 5. Testis-ova in shortnose sturgeon from Delaware River: Small cluster of ovarian tissue mixed with advanced testicular tissue projecting from an immature testis (a) consisting of encysted spermatogonia (b). Spermatic cysts in close proximity to testis-ova were frequently disorganized with reduced numbers of spermatogonia (c). Cysts intermingled within the testis-ova often contained a disorganized mix of germinal cells (d) or with mature

spermatozoa (e). OC, oocytes $_{(stage\ 1\text{-}6)}$; GN, oogonia; SG, spermatogonia; SZ, spermatozoa. Scale bar = 500 µm (a), 100 µm (b-e).

Fig. 6. Zonal distribution of intersex in shortnose sturgeon from Delaware River: adjacent bands of predominantly testicular and ovarian tissue (a). Few oocytes were located under the tunica albuginea in the testicular zone (b) and small areas of spermatozoa were restricted to the periphery of zones (c). Scale bar = 1 mm (a), 200 µm (b and c)

Fig. 7. Seasonal proportions of maturity stages and intersex condition in shortnose sturgeon collected from Delaware River, 2006-2011. O, ovo-testis; T, testis-ova; Z, zonal

Fig. 8. Total length and weight of shortnose sturgeon by reproductive maturity stage or intersex category. Boxes indicate 75th percentile (top line), median (middle line) and 25th percentile (bottom line) of data range, while error bars indicate extreme values. Sample sizes (n) are located above the x-axis. O, ovo-testis; T, testis-ova; Z, zonal

Fig. 9. Condition factors of shortnose sturgeon by reproductive maturity stage or intersex category. Boxes indicate 75th percentile (top line), median (middle line) and 25th percentile (bottom line) of data range, while error bars indicate extreme values. Sample sizes (n) are located above the x-axis. O, ovo-testis; T, testis-ova; Z, zonal

Fig. 11. Mean concentration of steroid hormones in shortnose sturgeon. Error bars indicate SEM and sample size is located over each pair of bars. O, ovo-testis; T, testis-ova; Z, zonal

Fig. 12. Testosterone:Estradiol ratio in shortnose sturgeon. Error bars indicate SEM and sample size is located over each bar. O, ovo-testis; T, testis-ova; Z, zonal

Fig. 1

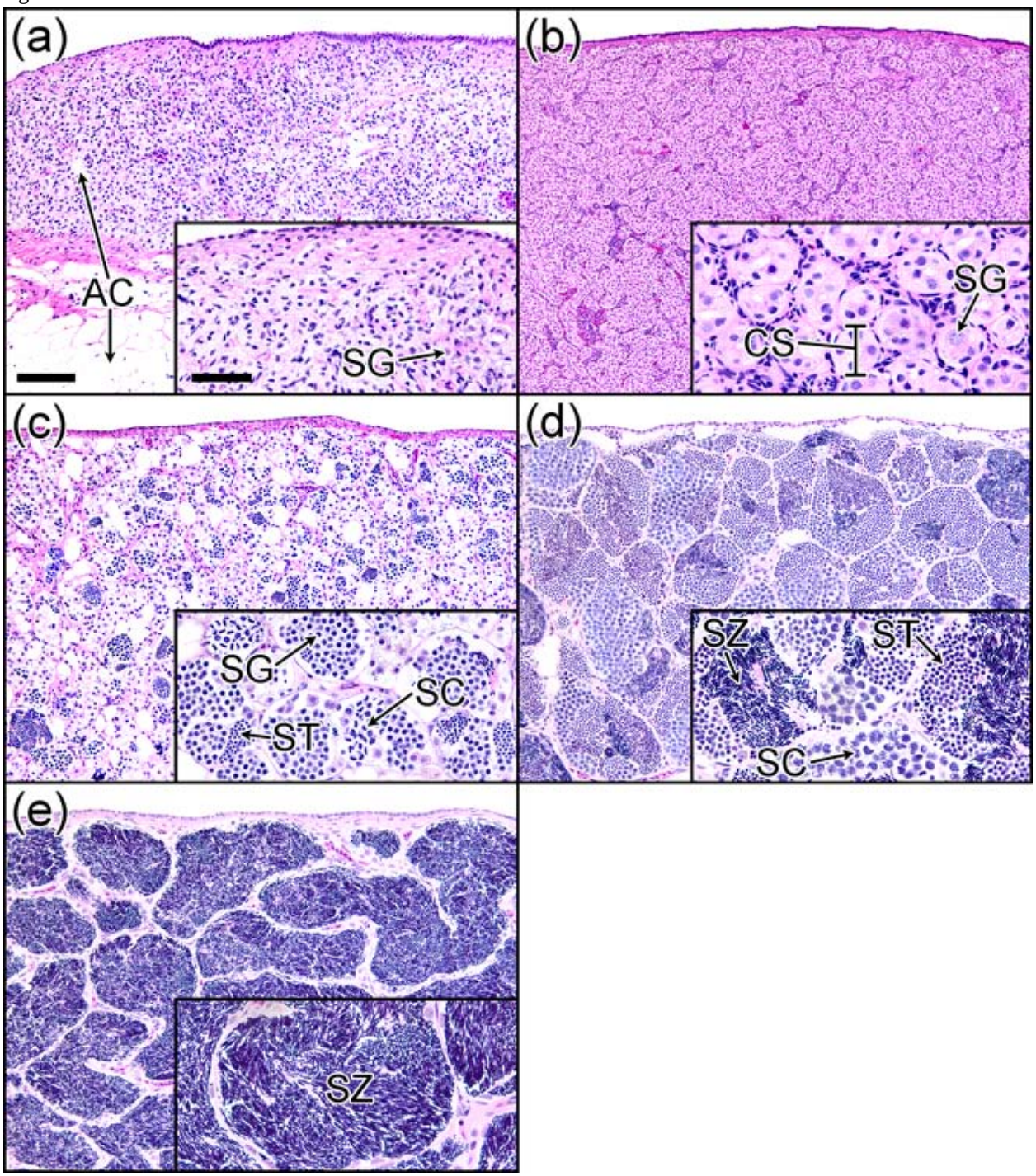
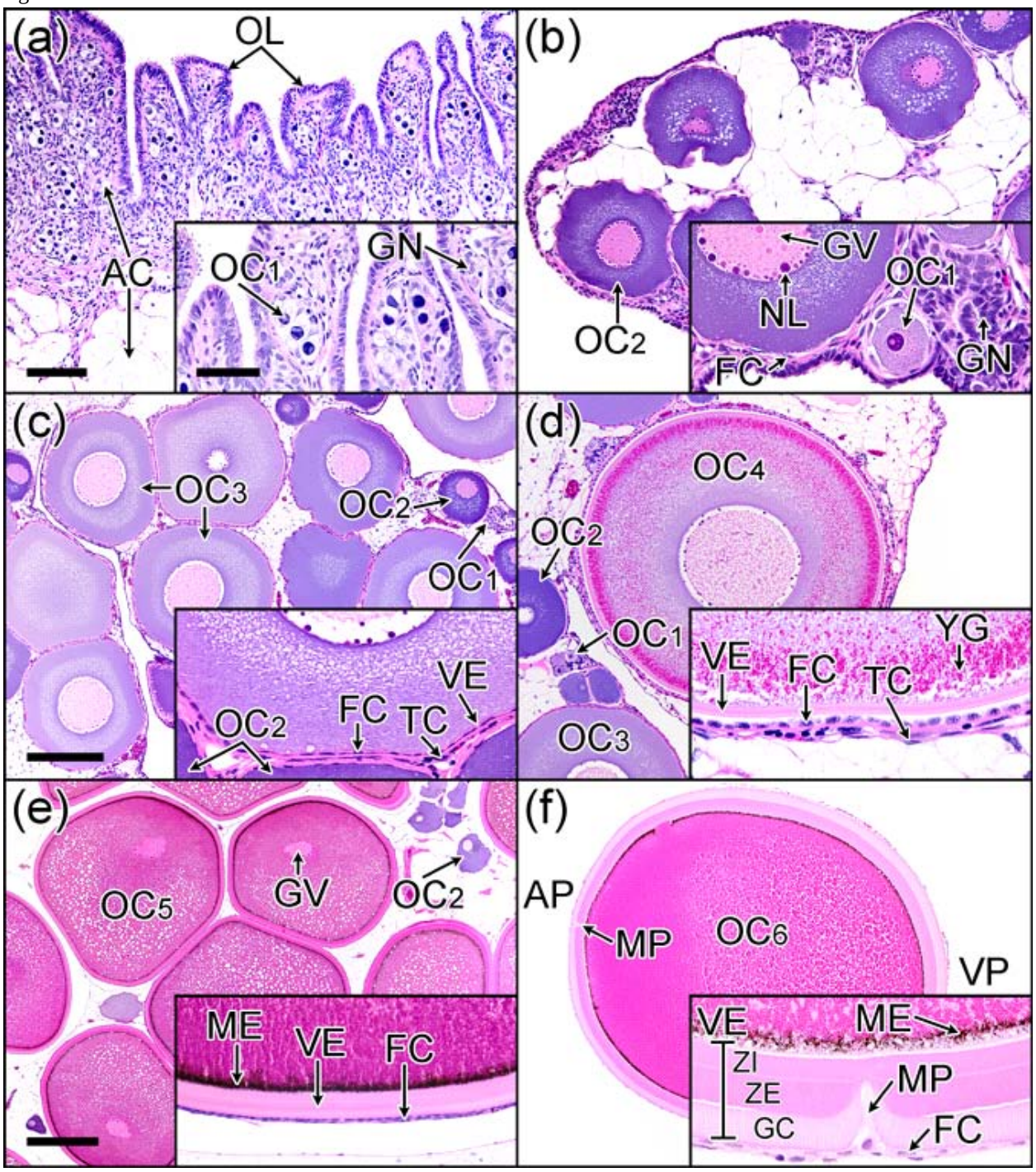


Fig. 2


(a)
OL
AC
OC1
GN
(b)
GV
OC1
NL
OC2
FC
GN
(c)
OC3
OC2
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VE
OC2
FC
TC
(d)
OC4
OC2
OC1
OC3
YG
VE
FC
TC
(e)
OC5
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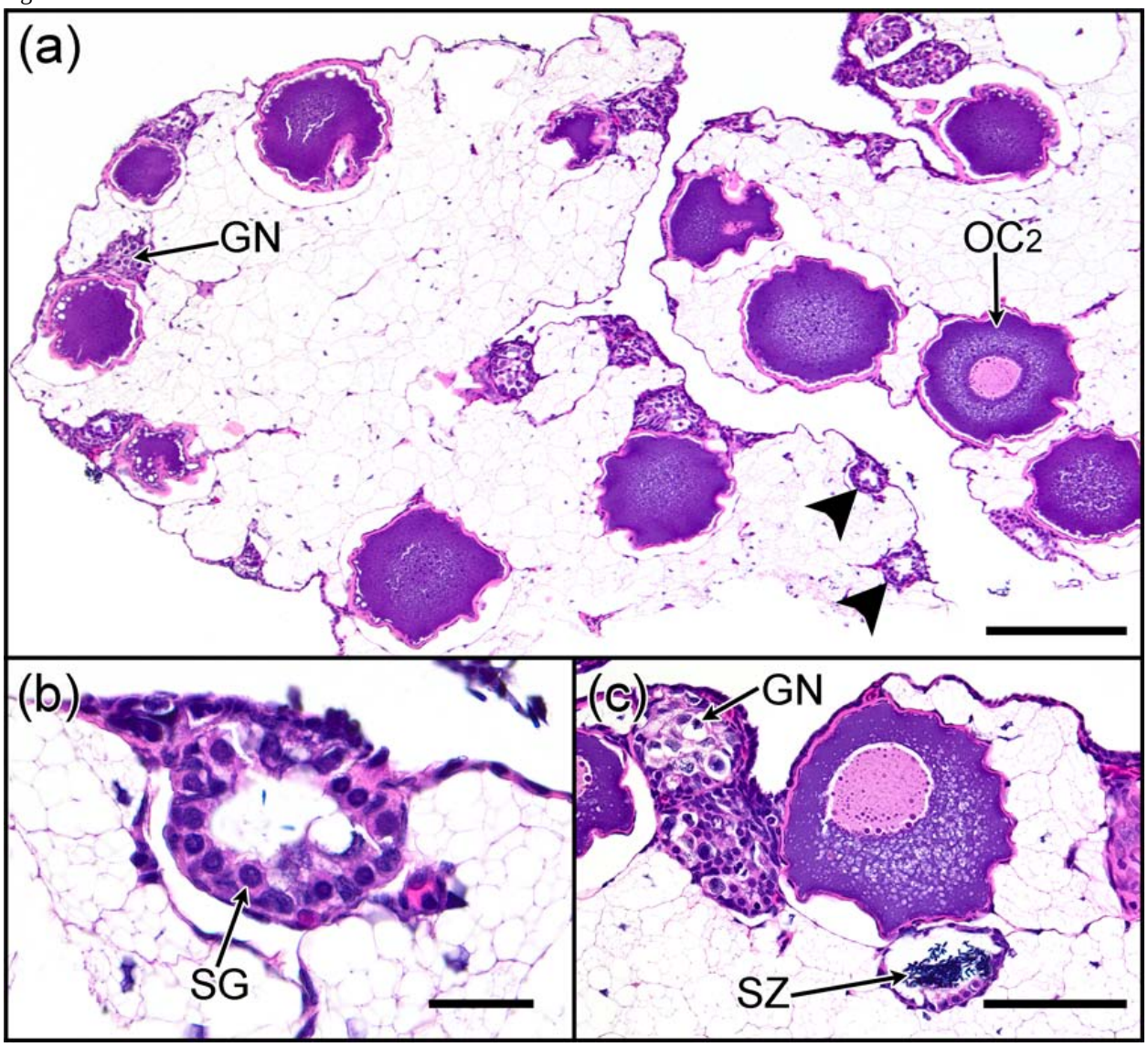
GC
MP
FC

Fig. 3



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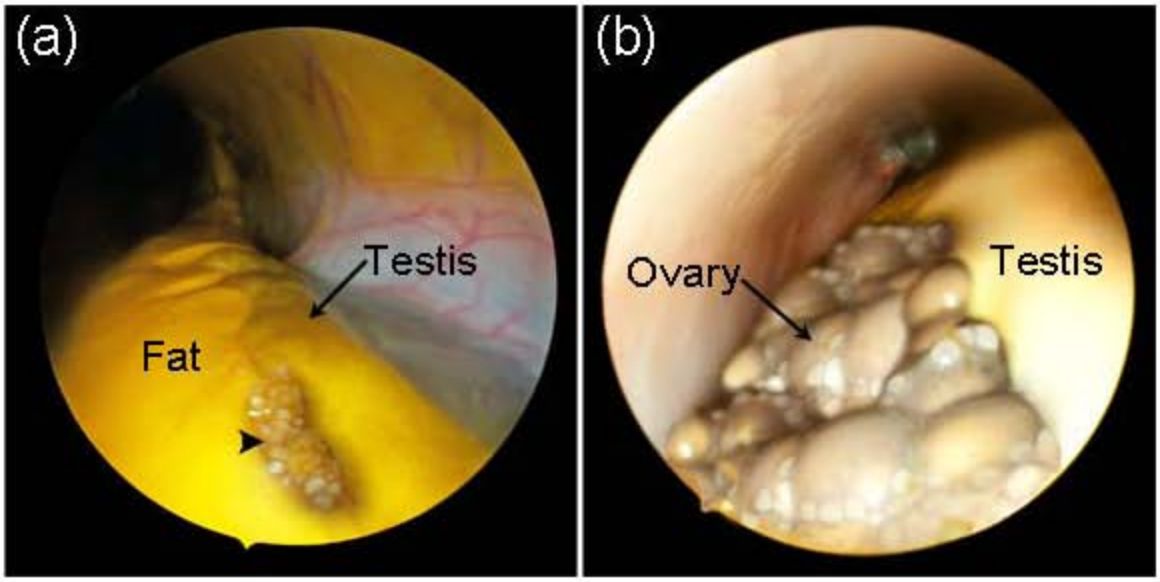

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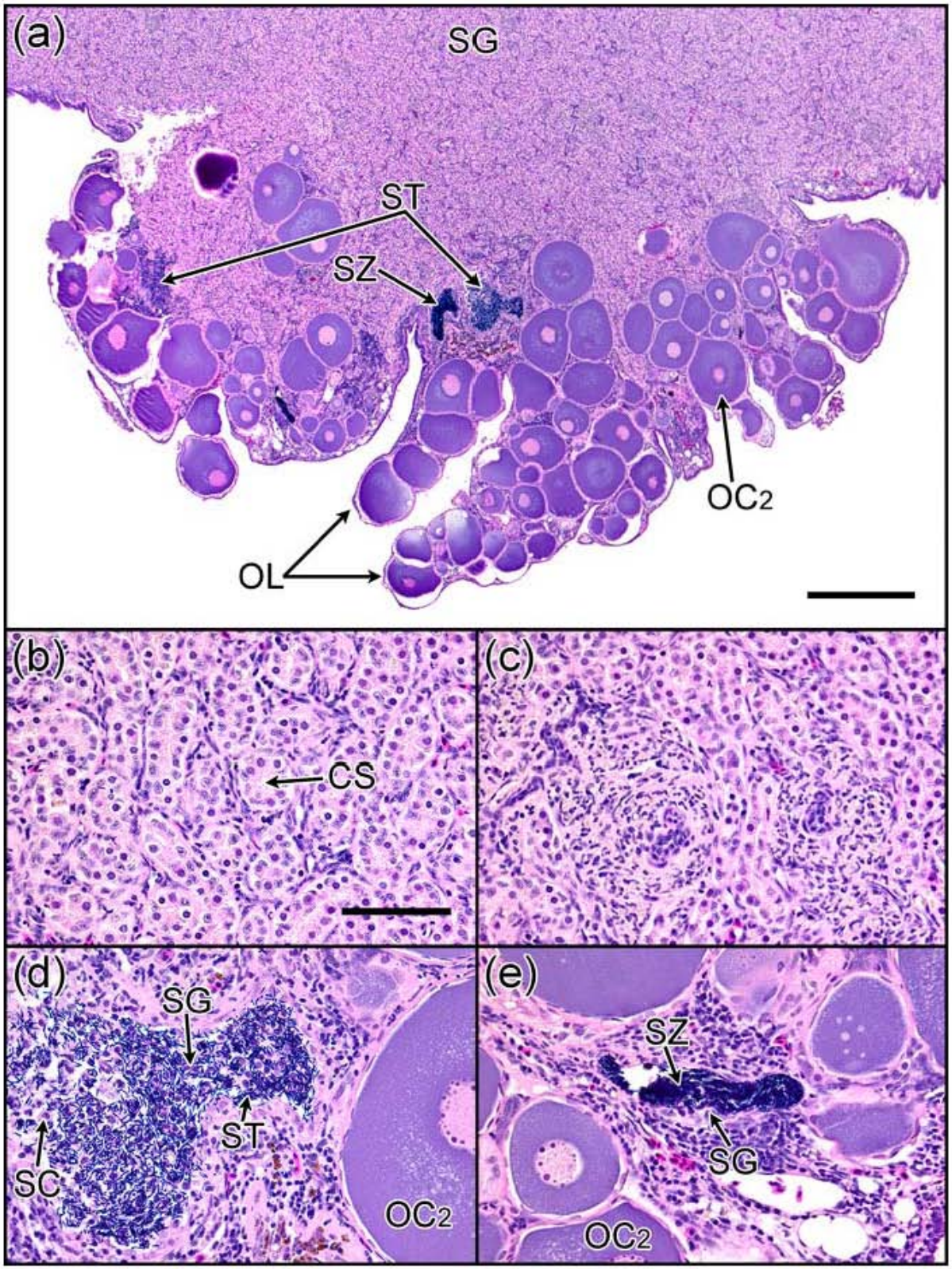

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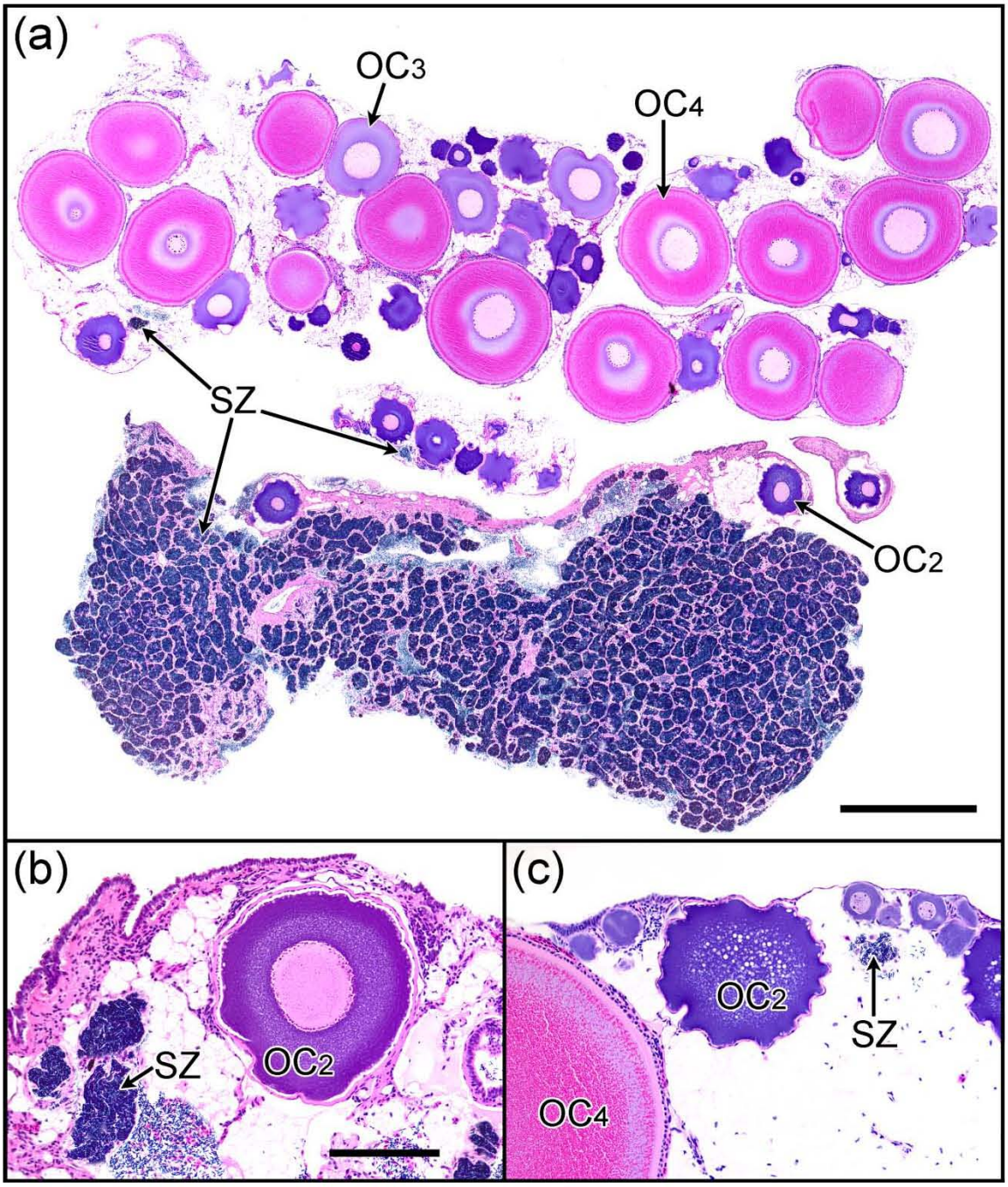

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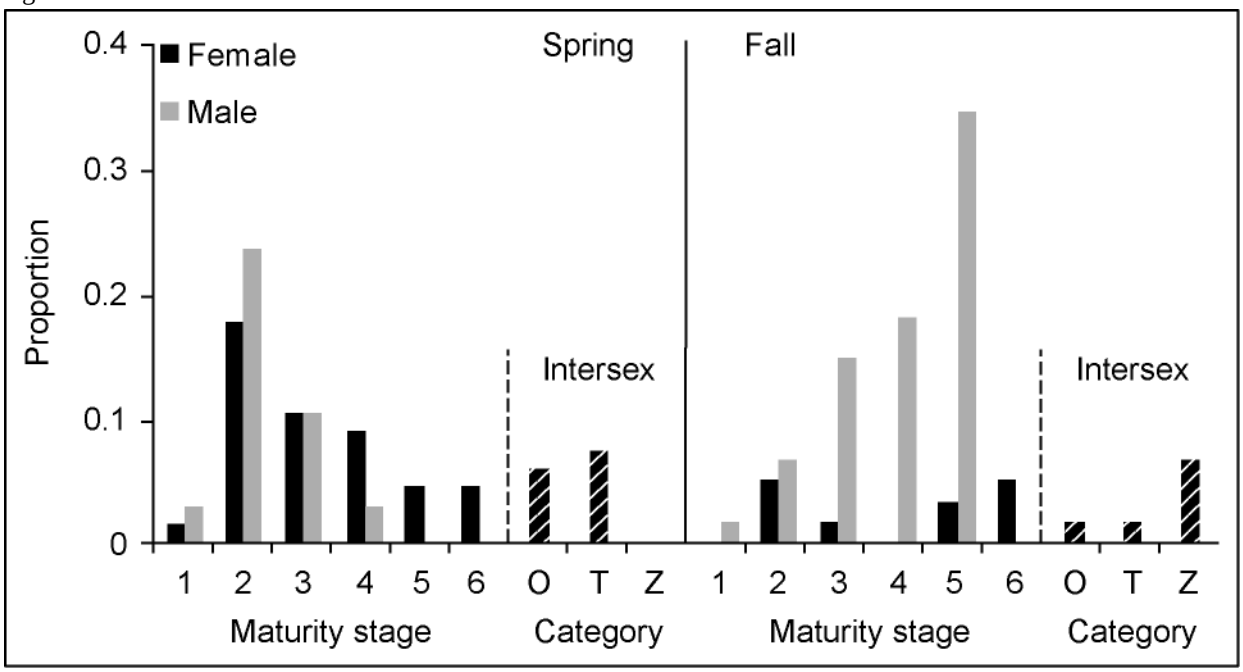
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0.2
0.1
0
Proportion
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Male
Spring
Fall
Intersex
Intersex
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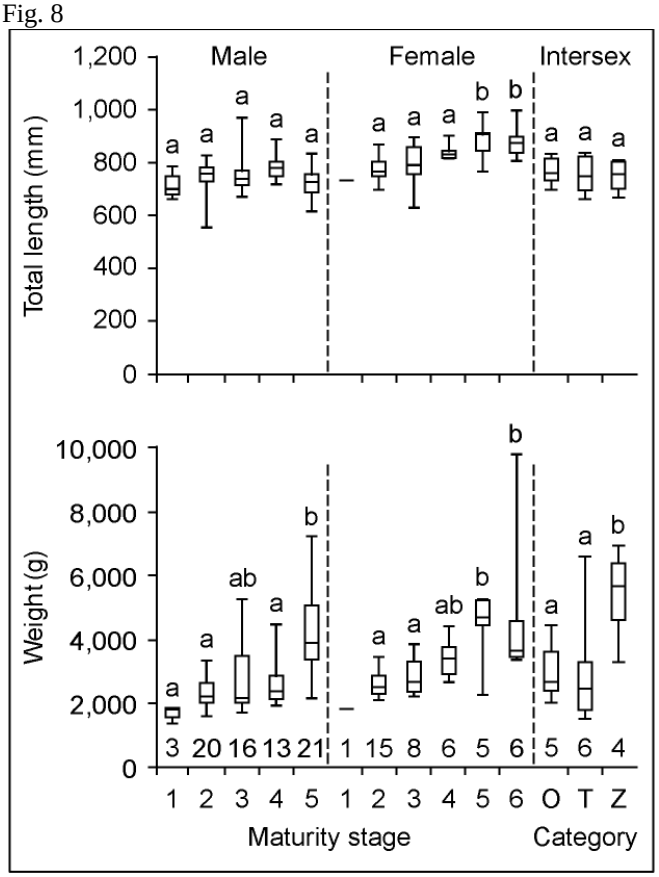
Maturity stage
Category
Maturity stage
Category

Fig. 8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6,000
4,000
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0
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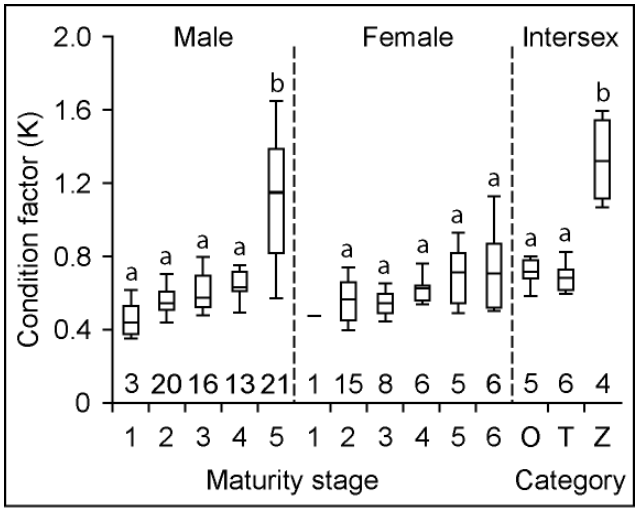
1 2 3 4 5 1 2 3 4 5 6 O T Z
Maturity stage
Category

Fig. 9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Female
Intersex
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1.2
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0.4
0
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1 2 3 4 5 1 2 3 4 5 6 O T Z
Maturity stage
Category

Fig. 10

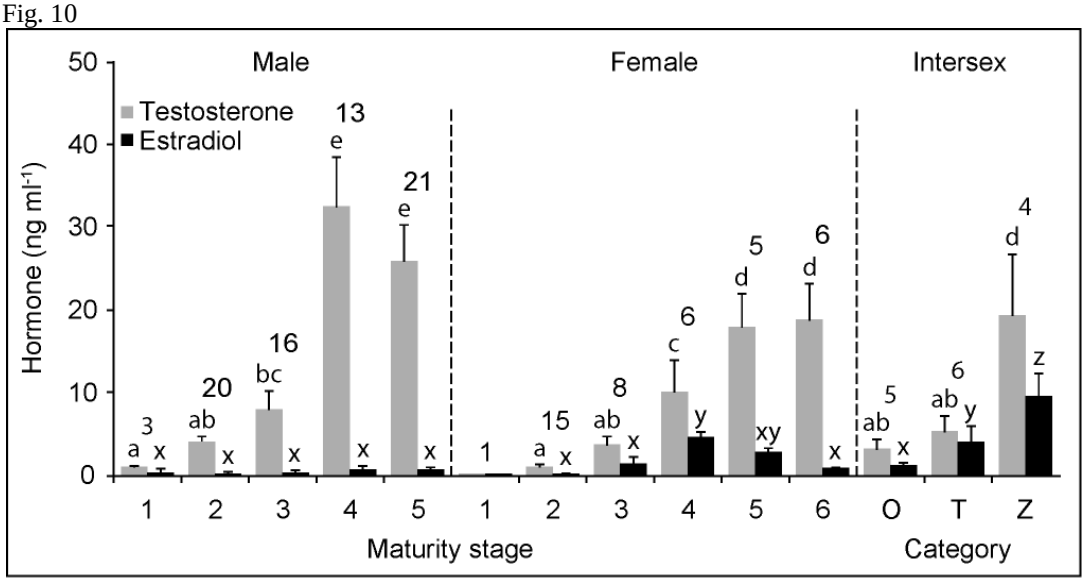
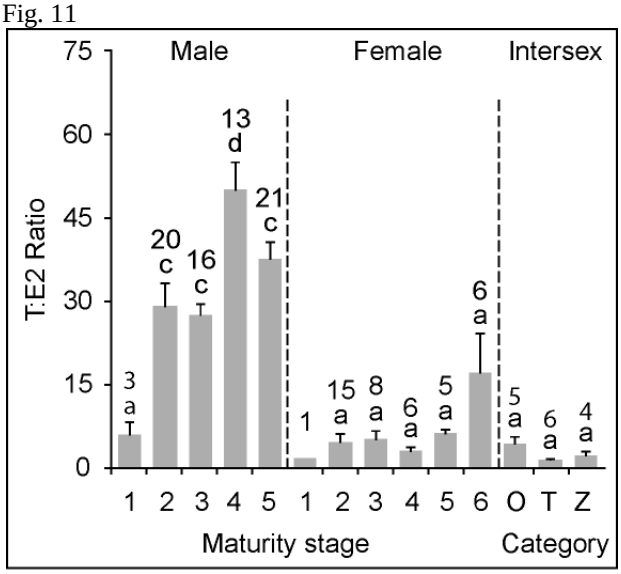

Male
Female
Intersex
Testosterone
Estradiol
Hormone (ng ml-1)
Maturity stage
Category
1 2 3 4 5 1 2 3 4 5 6 O T Z

Fig. 11


Male
Female
Intersex
T:E2 Ratio
Maturity stage
Category
1 2 3 4 5 1 2 3 4 5 6 O T Z

Chapter 2: Hematology and plasma chemistry of shortnose sturgeon from Delaware River, USA

By Mark A. Matsche[1], Kevin M. Rosemary[1], Harold M. Brundage III[2] and John C. O'Herron, II[3]

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Summary

The effects of gender, maturity, intersex condition and annual and seasonal variability on hematology and plasma chemistry of wild shortnose sturgeon *Acipenser brevirostrum* were studied. Fish were captured by gill net from the Delaware River as part of a general and reproductive health survey. A total of 68 fish were examined in May-June, and 61 additional fish were examined in November during 2006-2011. Total leukocyte counts (WBC), leukogram, PCV and 13 biochemical parameters were measured from these fish using standard clinical methods. Season and gender had no effect on hematologic indices, but PCV was inversely related to maturity of fish. Annual variation in neutrophil, eosinophil and monocyte counts was significant but WBC and neutrophil:lymphocyte ratios were not significantly affected. As was expected, there was a high degree of variability among biochemical analytes. Season had the greatest affect on sodium and chloride, and was likely related to environmentally induced changes in osmoregulation. Reproductive maturity had the greatest affect on plasma proteins, while the effects of gender and maturity were approximately equal on calcium and potassium. Glucose differed significantly by gender, possibly indicating different energetic requirements of males and females. Reference intervals for total protein and CPK were partitioned by reproductive maturity; albumin, sodium and chloride were partitioned by season and maturity; glucose was partitioned by gender and calcium was partitioned by gender and maturity of fish. None of the hematologic indices, globulin, potassium, urea, phosphorous, AST and lactate dehydrogenase met the criterion of reference interval partitioning by subcategory. Intersex condition was associated with lowered glucose, potassium, phosphorous and enzyme activities. Reference intervals reported here will be useful for evaluating the health and physiological condition of fish.

Introduction

Sturgeon populations worldwide are either endangered, threatened or at risk over exploitation (Pikitch *et al* 2005). With few signs of recovery, there is increased interest in hatchery propagation of sturgeons for conservation measures, including production of caviar and meat to reduce demand on wild stocks, and in stock assessment of remaining populations. Diagnostic tools such as hematologic and biochemical analysis, are essential as a quantitative measure of stress and physiological condition in animals. Changes in blood values are often indicative of changes in health condition or physiological state, and can be useful in identifying disease or assessing response to pollution, habitat degradation or other changing environmental conditions (Blaxhall 1972, Lockhart & Metner 1984). The use of blood value analysis in fish as a diagnostic tool however, has been limited by a lack of available reference intervals. Fish exhibit a tremendous variation in the numbers and types of blood cells and in the biochemical composition and regulation of the intravascular fluid (Hrubec & Smith 2000, Campbell 2006a, 2006b), which often requires species-specific reference intervals at a minimum for meaningful interpretation of hematologic and biochemical data.

In recent years, there has been increased interest in determining "normal" blood values from sturgeon for diagnostic reference. Reference intervals have been reported for captive shortnose sturgeon *Acipenser brevirostrum* (Knowles *et al* 2006), Amur sturgeon *A. schrenckii* (Shi *et al*. 2006), Chinese sturgeon *A. sinensis* (Shi *et al*. 2006), and sturgeon hybrids (*A. naccarii* female x *A. baerii* male, DiMarco *et al*. 2011). Additional studies provide normal blood values from captive or wild fish including *A. baerii* (Palíková *et al.* 1999), *Huso huso* (Palíková *et al.* 1999, Asadi *et al*. 2006a, Zarejabad *et al* 2010), *A.stellatus* (Palíková *et al.* 1999, Shahsavani *et al* 2010), Persian sturgeon *A. persicus* (Asadi *et al*. 2006b) and pallid sturgeon *Scaphirhynchus albus* (Jenkins 2003). These studies highlight the marked differences in blood values among closely related species (Shi *et al*. 2006). Reference intervals for shortnose sturgeon are limited to subadult (3-5 year old), hatchery reared fish (Knowles *et al*. 2006).

Hematologic and biochemical values in fish are highly influenced by a number of extrinsic and intrinsic factors including environmental (e.g. temperature, salinity and photoperiod) (Ram Bhaskar & Rao 1989, Hrubec *et al*. 1997a, Valenzuela 2006, Bani *et al*. 2009, Zarejabad *et al*. 2010, DiMarco *et al*. 2011); biological (e.g. age, sex, maturity and activity levels) (Blaxhall 1972, Hrubec *et al*. 2001, Baker *et al*. 2005, Asadi *et al*. 2006b) and anthropogenic (e.g. pollution, hypoxia or degraded habitat) (Swift 1981, Hrubec *et al*. 1997b, Huertas *et al*. 2002, Cazenave *et al*. 2005, Jerônimo, G.T. *et al.* 2009). In sturgeon, salinity (LeBreton & Beamish 1998, Jarvis &

Ballantyne 2003, Zarejabad *et al*. 2010), temperature (Sadati *et al*. 2011), gender and maturity (Asadi *et al*. 2006a, Asadi *et al*. 2006b, Shahsavani *et al* 2010a, Shahsavani *et al* 2010b) and handling and confinement stress (Barton *et al*. 2000, Kiefer *et al*. 2001) can have a significant influence on blood values. Therefore it may be necessary to further define reference intervals by gender, maturity, season, location or other sub-categories, to increase the usefulness of hematologic and biochemical analysis as a diagnostic tool. To that end, the goal of this study was to evaluate the effects of year, season, gender, maturity and intersex condition on hematologic and plasma chemistry values, and determine reference intervals for those blood values in a wild population of shortnose sturgeon. Reference intervals obtained in this study are relevant for future monitoring of health and condition of these fish, and may serve as a benchmark for evaluating the impacts of disease, deleterious environmental changes or management actions.

Methods

Blood samples were collected from fish that were assessed for gender and reproductive maturity (see Chapter 1). Total white cell count (WBC), PCV and leukogram were determined according to Knowles *et al* (2006). Heperanized blood was centrifuged at 10,000 x g for 5 min, and plasma was decanted and placed on ice for storage and transport. Glucose, urea, total protein, albumin, aspartate aminotransferase (AST), calcium, phosphorous, sodium, potassium, chloride, globulin, creatinine phosphokinase (CPK) and lactate dehydrogenase (LDH) were measured from plasma using an automated chemistry system (AU-5400, Olympus America Inc.).

Hematology and plasma chemistry data were tested for normal distribution (Kolmogorov-Smirnov) and homoscedasticity (Bartlett). Urea, albumin, calcium, potassium and CPK were normalized by $\log_{10}$ transformation. Prior to analysis, data were binned according to the following maturity categories: immature = stage 1 and 2 females and stage 1 and 2 males; developing = stage 3 and 4 females and stage 3 and 4 males; and mature = stage 5 and 6 females and stage 5 males. See chapter 1 for developmental stages of ovary and testis and intersex condition categories. Intersex conditions identified were: ovo-testis, spermatic cyst-like structures within primarily immature ovary; testis-ova, foci of ovarian lamellae in predominantly immature testis; and zonal distribution of intersex tissues, consisting of pockets of either mature ovarian or testicular tissues scattered along the gonad. For statistical analysis, ovo-testis was categorized as immature, testis-ova was categorized as developing and zonal was categorized as mature. Pearson correlation coefficient was used with transformed and non-transformed data to test

the effects of year, season (spring and fall), gender (male, female and intersex) and maturity of fish (immature, developing and mature) on hematology and plasma chemistry analytes.

Reference intervals were determined for non-intersexual fish by robust estimation methods following the CLSI guidelines (Horn & Pesce 2005, CLSI 2008). Robust methods are recommended for reference interval determination when fewer than 120 samples are available for analysis (CLSI 2008). Statistical differences in blood values do not necessarily indicate clinical significance. Therefore each analyte was checked for partitioning by subclass (season, sex and maturity category) according to the means comparison method of Sinton *et al*. (1986). When the Pearson correlation coefficient was statistically significant, reference intervals were partitioned by subclass if the maximum difference in mean values was at least 25% of the reference interval estimated from the combined data. Statistical analysis was performed using SAS Enterprise Guide 4.1 (Davis 2007).

Results

The morphology and size of blood cells in shortnose sturgeon examined in this study were similar to those reported in subadult shortnose sturgeon reared in captivity (Knowles *et al*. 2006). All fish were apparently healthy and there were no indications of hematological disorders or blood-borne parasites.

Neutrophil, eosinophil and monocyte counts varied significantly from year-to-year (Table 1). There was a significant inverse relationship between PCV and maturity of fish, although no effect of sex was detected (Table 1, Fig. 1).

Plasma proteins (total protein, albumin and globulin) and urea varied significantly by season and maturity of fish (Table 2). Proteins were highest in mature fish and in the fall, while urea was highest in immature fish and in the spring (Fig. 2). Glucose varied significantly by sex (Table 2.), with the lowest values occurring in intersex fish (Fig. 2). Sodium and chloride varied significantly by year, season and maturity of fish (Table 2). Plasma potassium and phosphorus varied significantly by season, sex and maturity (Table 2). Potassium and phosphorus were highest in females and in the spring, were lowest in intersex fish and in the fall (Fig. 2), and were inversely related to maturity (Table 2). Calcium and CPK varied significantly by sex and maturity of fish (Table 2). CPK was highest in mature females and lowest in intersex fish, while calcium was highest in mature females and lowest in immature males (Fig. 2). There was a significant effect of season and sex on AST (Table 2.). AST was highest in males in spring and lowest in intersex fish in fall (Fig. 2).

Reference intervals are presented for hematology (Table 3) and plasma chemistry (Table 4) analytes. None of the hematology analytes met the criterion for partitioning. Albumin, glucose, sodium and chloride were partitioned by season; glucose and calcium were partitioned by gender; and total protein, albumin, sodium, chloride and CPK were partitioned by maturity status (Table 4).

Discussion

PCV is perhaps the most widely reported hematological index in fishes, and is a useful indicator of health and physiological condition (Hrubec & Smith 2000). The normal range in PCV can vary considerably in individuals and within and among species. Age, sex, maturity state, water temperature, photoperiod and season can affect PCV values (Campbell 2006b). Disease, poor nutrition, crowding, exposure to toxicants, hypoxia and gonadal development can result in decreased PCV (Blaxhall 1972, Clauss *et al.* 2008). Changes in environmental conditions can often affect osmoregulation in fishes, resulting in a net influx or efflux of intravascular water (Olson 1992). Hemodilution or hemoconcentration of the blood may cause an apparent increase or decrease in PCV and blood cell counts (Noga 2000, Clauss *et al.* 2008). In this study, there was an inverse relationship between PCV and maturity of fish. The reference interval for PCV (24-40) in this study, which included immature through maturity stages, was generally comparable but slightly low compared to that of immature, cultured shortnose sturgeon (26-46, Knowles *et al*. 2006). Differences in water quality conditions and types of stressors encountered by wild and cultured fish can lead to significant differences in blood cell values (Hrubec *et al.* 1996).

Reference intervals for WBC and leukocytes in wild shortnose sturgeon examined in this study were low compared to cultured shortnose sturgeon (Knowles *et al*. 2006). Mean WBC in this study was about half of the reported WBC for cultured shortnose sturgeon, and consequently, the reference ranges of most comparable leukocytes were also low. Mean small and large lymphocytes, neutrophils and monocytes were 12-83% lower than mean values of those leukocytes in cultured shortnose sturgeon (Knowles *et al*. 2006). However, mean eosinophil counts and mean N:L ratios were 73% and 52% higher, respectively, in this study than in that of Knowles *et al.* (2006). In contrast, mean WBC, lymphocyte, neutrophil and monocyte values reported here for wild shortnose sturgeon were higher than the mean values for those analytes in cultured, juvenile *H. huso* (Zarejabad *et al*. 2010). As with PCV, leukocyte counts can vary by season, environmental conditions (e.g. temperature and salinity), culture conditions, disease and exposure to toxicants (Noga 2000). Therefore the differences in WBC and leukogram

evident among the few available studies of sturgeon hematology are not surprising, and highlight the need for well defined, species-specific reference intervals.

Hematologic reference intervals for cultured shortnose sturgeon (Knowles *et al*. 2006) were generally much wider than those obtained in this study. It is possible that cultured, immature shortnose sturgeon exhibit a greater degree of variability in hematologic values than in wild fish. Fish held in captivity may be subject to additional stressors such as handling, crowding and potentially unsuitable enclosures or enclosure materials (e.g. fiberglass tanks). However, blood values in fish are also affected by age, gender and maturity. Fish examined in this study were more diverse in maturity, and likely in age, than the cultured shortnose sturgeon used in the study of Knowles *et al.* (2006). Wider reference intervals reported by Knowles *et al.* (2006), in comparison to this study, may be related to differences in techniques used to calculate reference intervals. Robust methods were used to calculate reference intervals in this study, whereas Knowles *et al.* (2006) used nonparametric techniques, which are recommended when sample size is sufficiently large (≥ 120 animals, CLSI 2008). However, hematologic reference intervals reported by Knowles *et al.* (2006) were based on examination of 46 fish. Nonparametric techniques may be unduly influenced by outlying observations when sample sizes are small, and may result in excessively wide reference intervals, particularly at the upper limit (Horn & Pesce 2005).

Seasonal differences in biochemistry may result from the wide range of environmental conditions that fish encounter over the course of a year (McDonald & Milligan 1992). Temperature, salinity, photoperiod and other environmental conditions that may change seasonally are known to affect metabolism (McDonald & Milligan 1992). In this study, most biochemical analytes were significantly affected by season. In general, proteins and most electrolyte levels were higher in the fall in this study, while urea, glucose, potassium and phosphorous levels were higher in the spring. Plasma proteins are mainly altered by changes in plasma volume, which often result from increased or decreased gill permeability (McDonald & Milligan 1992). Decreased proteins and electrolytes in spring may indicate a hemodilution effect related to seasonal changes in osmoregulation (McDonald & Milligan 1992). In yellowtail flounder *Limanda ferruginea*, osmolality and sodium were reduced during the spring and were negatively correlated with dissolved oxygen levels (Mercaldo-Allen *et al.* 2003). The authors suggested that reduced osmolality in spring may be related to increased dissolved oxygen levels following spring turnover (Mercaldo-Allen *et al.* 2003).

Potassium, urea, glucose and phosphorous were higher in the spring than in the fall in this study. Potassium levels were significantly lower in winter and fall than in summer in yellowtail flounder (Mercaldo-Allen *et al.* 2003) and in the windowpane *Scophthalmus aquosus* (Dawson 1990). In contrast to this study, potassium levels in the Eurasian perch were lowest in the spring than in the fall (Sandstrom 1989). Hypokalemia may occur at lower temperatures following a decline in metabolism and feeding (Umminger & Mahoney 1972, Bentnick-Smith *et al.* 1987), while hyperkalemia may result from mobilization of potassium from skeletal muscle or erythrocytes following increased exercise or stress (McDonald & Milligan 1992). Glucose is often used as a stress indicator and is affected by many factors, diet being perhaps the most important (McDonald & Milligan 1992). In the tench *Tinca tinca* glucose was higher in the fall than in the spring (Guijarro *et al*. 2003), which the authors attributed to increased food availability in the fall. Little is known about regulation of urea and phosphorous in fish. Other than sharks, most fish produce little urea and most excretion is across the gills, indicating that changes in plasma urea are probably linked to differences in gill function (McDonald & Milligan 1992). In shortnose sturgeon from the Delaware River, elevated urea, glucose and potassium levels in the spring (late-May and early June) may indicate increased activity and feeding with increased water temperature in comparison to fall (early November) when temperatures begin to decline.

Gender and maturity can have a large impact on metabolism and osmoregulaton in fish, resulting in a variety of intravascular changes (Sandstrom 1989, Luskova 1998, Guijarro *et al.* 2003). Glucose and AST were significantly higher in females in this study, but were not affected by maturity status. Shahsavani *et al.* (2010a) did not detect a difference in glucose by gender in starry sturgeon, but Asadi *et al.* (2006a) reported significantly higher glucose in male *H. huso*. AST was higher female *A. stellatus* (Shahsavani *et al.* 2010b) and increased with maturity in female *A. persicus* (Asadi *et al.* 2006b). Protein levels, sodium, chloride and urea increased with maturity in both sexes in this study. Protein values were higher in male starry sturgeon and *H. huso* (Asadi *et al*. 2006a, Shahsavani *et al*. 2010a). Sodium was significantly higher in female starry sturgeon (Shahsavani *et al.* 2010a). Calcium, potassium, phosphorous and CPK levels were higher in females and were affected by maturity in this study. Calcium is correlated to circulating vitellogenin levels, and generally increases with maturity in females (Linares-Casenave *et al.* 2003). However, differences in calcium were not detected by gender in *H. huso* (Asadi *et al.* 2006a) and in mature starry sturgeon (Shahsavani *et al.* 2010a), or by gender or maturity in *A. persicus* (Asadi *et al.* 2006b). Phosphorous was significantly higher in male *H. Huso* (Asadi *et al.* 2006a), but was higher in female starry sturgeon

(Shahsavani *et al.* 2010a). Creatinine kinase was also significantly higher in *A. persicus* (Asadi *et al.* 2006b) and *A. stellatus* (Shahsavani *et al.* 2010b), and decreased with maturity in female *A. persicus* (Asadi *et al.* 2006b), as it did in shortnose sturgeon in this study.

Affect of year on biochemistry of fish was limited to sodium and chloride, which are considered to be sensitive to a wide variety of environmental and endogenous influences (McDonald & Milligan 1992). Year of sampling also had a significant effect on neutrophils, eosinophils and monocytes, which can vary in response to a variety of stressors that may change in intensity and frequency from year-to year (Noga 2000). Years with extremes in weather (e.g. temperature and precipitation) may have some influence on hematologic or biochemical values in fish, but overall, annual fluctuations in blood values appear to be relatively unimportant.

This study provides a detailed profile on blood values from a wild population of shortnose sturgeon. Diverse environmental conditions encountered in the wild and physiological changes that accompany the reproductive cycle can have a large impact on the hematology and biochemistry in fish. Reference intervals as a diagnostic tool are more useful when partitioned by physiologically important subcategories, such as gender, maturity and season.

Acknowledgements

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Table 1.
Pearson correlation coefficients (PCC) between hematologic analytes and reproductive state of fish, season (spring and fall) and year of sampling. N = 114.

		PCV	WBC	Large lymphocytes	Small lymphocytes	Neutrophils	Eosinophils	Monocytes	N:L ratio*
Year	PCC	0.00942	0.11912	0.10443	0.26303	-0.31694	0.45697	0.36932	-0.24313
	P	0.9163	0.1840	0.2445	0.0929	0.0003	<.0001	<.0001	0.1161
Season	PCC	-0.03236	0.01674	-0.08215	0.00076	0.00379	0.13073	0.00201	-0.07655
	P	0.7180	0.8524	0.3605	0.9932	0.9664	0.1445	0.9822	0.3943
Sex	PCC	0.05801	0.09403	0.08686	0.18302	-0.08397	0.05930	0.10587	-0.15706
	P	0.5171	0.2950	0.3335	0.0702	0.3499	0.5095	0.2381	0.0790
Maturity	PCC	-0.20072	-0.00100	0.06568	0.03477	-0.09099	0.13260	0.09272	0.00896
	P	0.0237	0.9912	0.4650	0.6991	0.3109	0.1388	0.3018	0.9206

*Lymphocytes = sum of small and large lymphocytes.

Table 2
Pearson correlation coefficients (PCC) between plasma chemistry analytes and reproductive state of fish, season (spring and fall) and year of sampling. N = 114. CPK, creatinine phosphokinase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase

		Total protein	Albumin	Globulin	Glucose	Sodium	Chloride	Calcium	Potassium
Year	PCC	0.01049	0.01722	0.01602	0.15147	-0.43445	-0.28362	-0.05986	0.12009
	P	0.9064	0.8476	0.8581	0.0892	<.0001	0.0012	0.5055	0.1787
Season	PCC	0.33381	0.50935	0.20394	-0.11191	0.72774	0.64519	0.07161	-0.56017
	P	0.0001	<.0001	0.0215	0.2103	<.0001	<.0001	0.4255	<.0001
Sex	PCC	0.01414	-0.06328	0.17318	-0.17590	0.09861	0.19813	-0.31733	-0.30785
	P	0.8741	0.4797	0.0515	0.0479	0.2700	0.1256	0.0003	0.0004
Maturity	PCC	0.50035	0.59070	0.31688	-0.14854	0.34035	0.35723	0.30377	-0.32737
	P	<.0001	<.0001	0.0003	0.0956	<.0001	<.0001	0.0005	0.0002

Table 2 continued.

		Urea	Phosphorus	CPK	AST	LDH
Year	PCC	0.15781	0.10364	-0.03491	0.10308	0.33761
	P	0.0752	0.2463	0.6968	0.2507	0.0961
Season	PCC	-0.39172	-0.40490	0.09562	-0.45828	-0.07349
	P	<.0001	<.0001	0.2849	<.0001	0.4116
Sex	PCC	-0.02597	-0.18263	-0.18856	-0.27993	-0.01069
	P	0.7711	0.0399	0.0338	0.0037	0.9050
Maturity	PCC	-0.22628	-0.32340	0.44639	-0.15490	0.05373
	P	0.0102	0.0002	<.0001	0.0833	0.5486

Table 3
Hematology reference intervals for shortnose sturgeon from the Delaware River. N =114.

			Robust interval	
Analyte	Mean	SD	Lower limit	Upper limit
PCV (%)	33	5.1	24	40
WBC (cells μL^{-1})	32,517	13,162	25,180	42,090
Small lymphocytes (cells μL^{-1})	12,539	6,770	14,420	17,251
Large lymphocytes (cells μL^{-1})	1,263	810	841	1,890
Neutrophils (cells μL^{-1})	9,817	6,265	6,250	12,660
Monocytes (cells μL^{-1})	480	410	352	635
Eosinophils (cells μL^{-1})	1,980	2,117	167	1,553
Neutrophil:lymphocyte ratio	0.85	0.95	0.39	1.05

Table 4
Plasma chemistry reference intervals for shortnose sturgeon from the Delaware River.

							Robust interval	
Analyte	Season	Sex	Maturity	Mean	SD	n	Lower limit	Upper limit
Total protein (g dl^{-1})	Combined	Combined	Immature	3.6	0.7	39	3.0	4.2
			Developing	4.4	1.0	43	3.4	5.6
			Mature	4.9	0.7	32	4.4	5.4
Albumin (g dl^{-1})	Spring	Combined	Immature	0.9	0.3	31	0.7	1.2
			Developing	1.0	0.5	21	0.7	1.3
			Mature	1.6	0.5	5	1.2	1.9
	Fall	Combined	Immature	1.0	0.5	8	0.7	1.4
			Developing	1.3	0.3	21	0.9	1.5
			Mature	1.8	0.4	26	1.4	2.2
Globulin (g dl^{-1})	Combined	Combined	Combined	3.3	0.9	114	2.6	4.0
Glucose (mg dl^{-1})	Combined	Male	Combined	88.7	30.7	73	55	110
		Female	Combined	78.7	4.5	41	48	93
Sodium (mmol L^{-1})	Spring	Combined	Immature	135	4.9	31	127	141
			Developing	136	3.8	21	130	141
			Mature	138	3.4	5	132	142
	Fall	Combined	Immature	147	4.5	8	140	154
			Developing	145	4.4	21	139	151
			Mature	144	3.6	26	139	149

Table 4 continued

Chloride (mmol L^{-1})	Spring	Combined	Immature	118	4.7	31	113	124
			Developing	116	5.1	21	110	122
			Mature	113	6.4	5	107	120
	Fall	Combined	Immature	125	5.0	8	118	131
			Developing	123	5.8	21	116	130
			Mature	121	4.7	26	116	127
Calcium (mg dl^{-1})	Combined	Male	Immature	8.2	0.7	23	7.4	8.8
			Developing	8.9	0.9	29	7.6	9.4
			Mature	8.7	0.7	21	7.5	9.1
		Female	Immature	9.9	3.0	16	7.3	12.4
			Developing	11.4	4.3	13	7.9	15.1
			Mature	17.4	6.0	9	8.2	26.3
Potassium (mmol L^{-1})	Combined	Combined	Combined	3.0	0.6	114	2.6	3.5
Urea (mg dl^{-1})	combined	Combined	Combined	2.4	1.1	114	1.5	3.2
Phosphorus (mg dl^{-1})	Combined	Combined	Combined	12.1	3.2	114	9.1	14.8
CPK[1] (U L^{-1})	Combined	Combined	Immature	1,569	973	39	280	288
			Developing	2,385	1,569	43	263	299
			Mature	4,260	1,638	32	258	266
AST[2] (U L^{-1})	Combined	Combined	Combined	483	164	114	357	617
LDH[3] (U L^{-1})	Combined	Combined	Combined	1,285	461	114	923	1,421

[1] creatinine phosphokinase
[2] aspertate aminotransferase
[3] lactate dehydrogenase

Figure Captions

Fig. 1. Hematology of shortnose sturgeon from Delaware River. For each analyte, the following letters above the plot indicate a significant effect: y = year, s = season, g = sex, m = maturity, no letter = no significant effects detected.

Fig. 2. Plasma chemistry of shortnose sturgeon from Delaware River. For each analyte, the following letters above the plot indicate a significant effect: y = year, s = season, g = sex, m = maturity, no letter = no significant effects detected. CPK, creatinine phosphokinase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase

Fig. 1

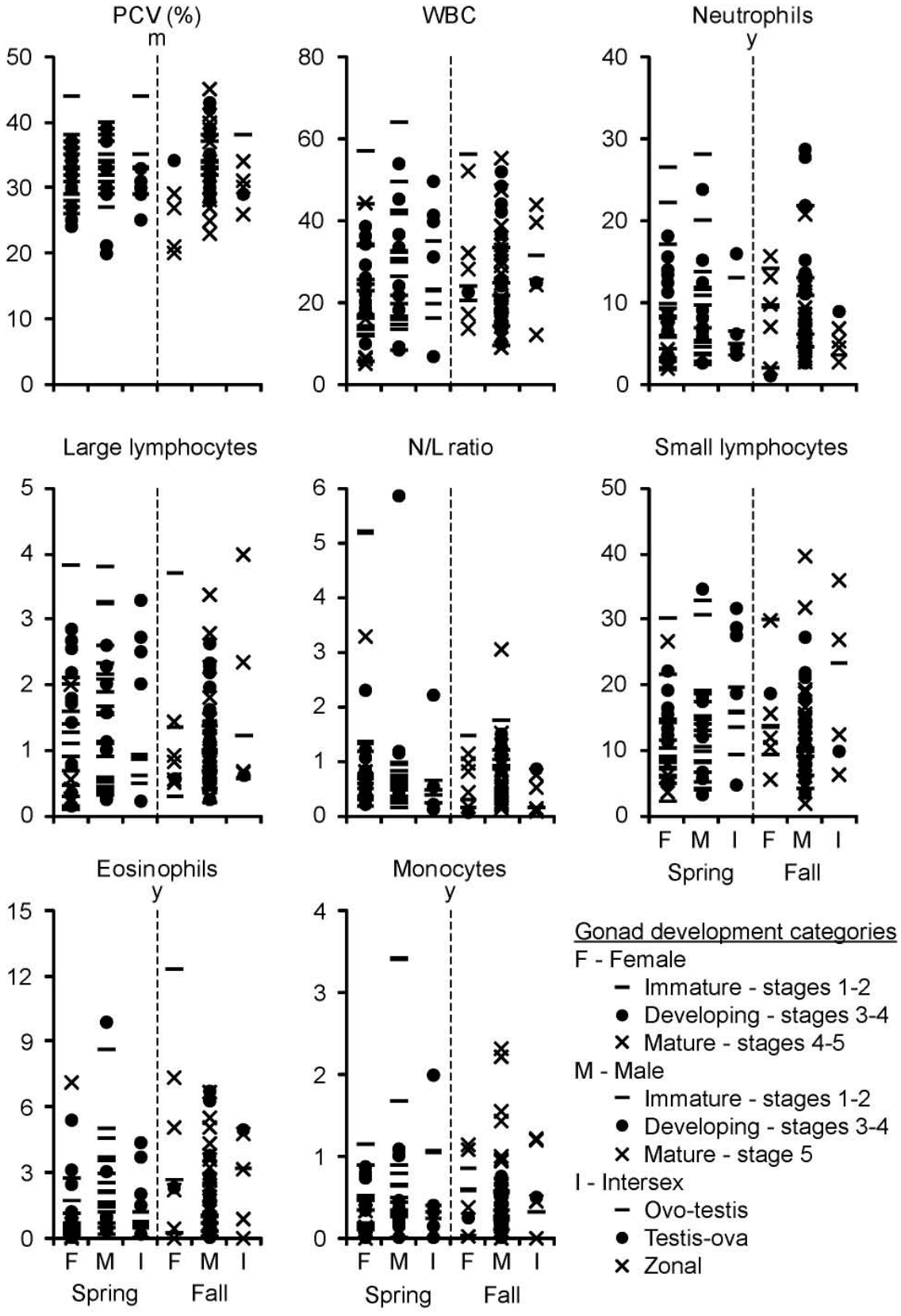

PCV (%)
m
WBC
Neutrophils
y
Large lymphocytes
N/L ratio
Small lymphocytes
Eosinophils
y
Monocytes
y
F M I F M I
Spring Fall
Gonad development categories
F - Female
Immature - stages 1-2
Developing - stages 3-4
Mature - stages 4-5
M - Male
Immature - stages 1-2
Developing - stages 3-4
Mature - stage 5
I - Intersex
Ovo-testis
Testis-ova
Zonal

Fig. 2

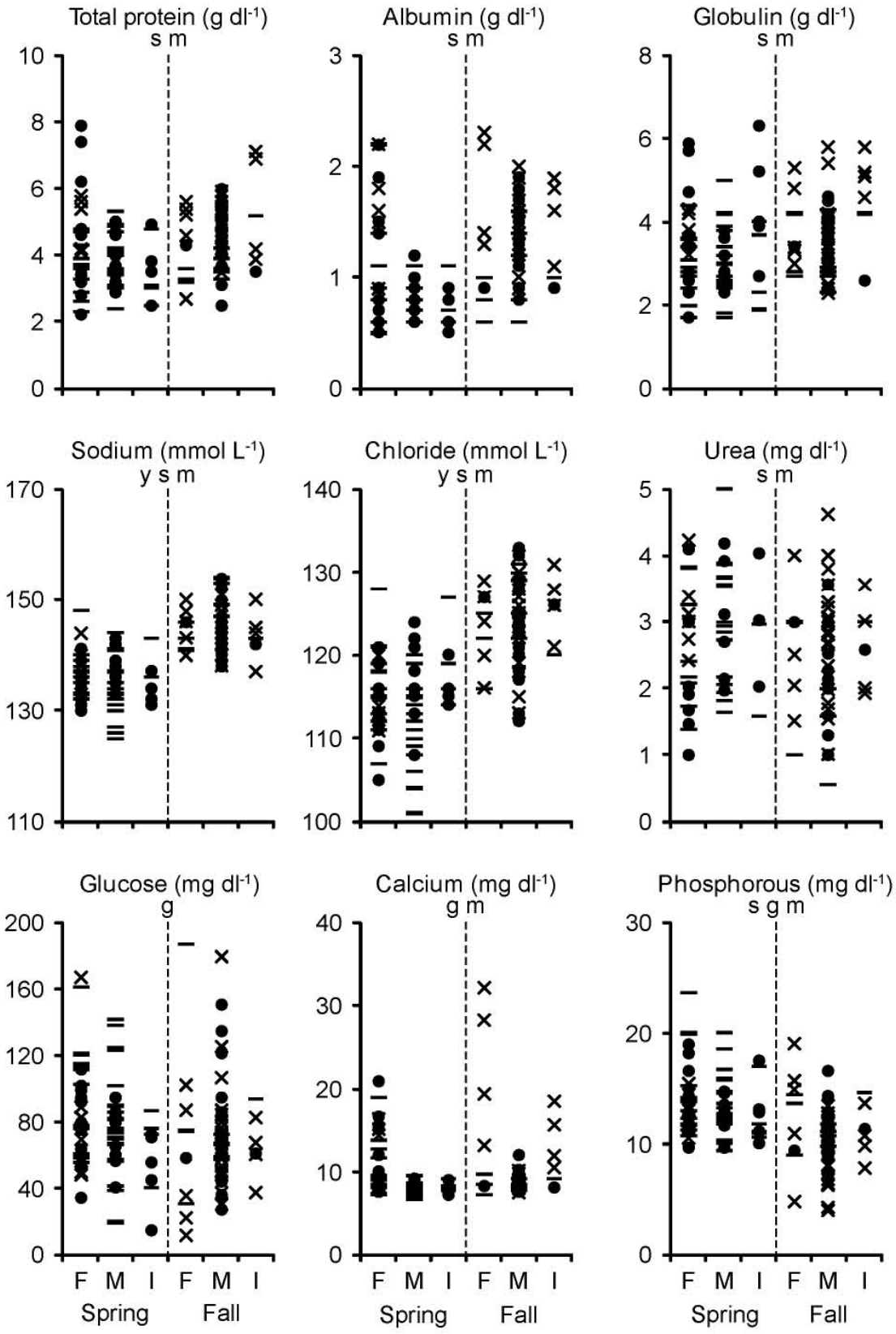
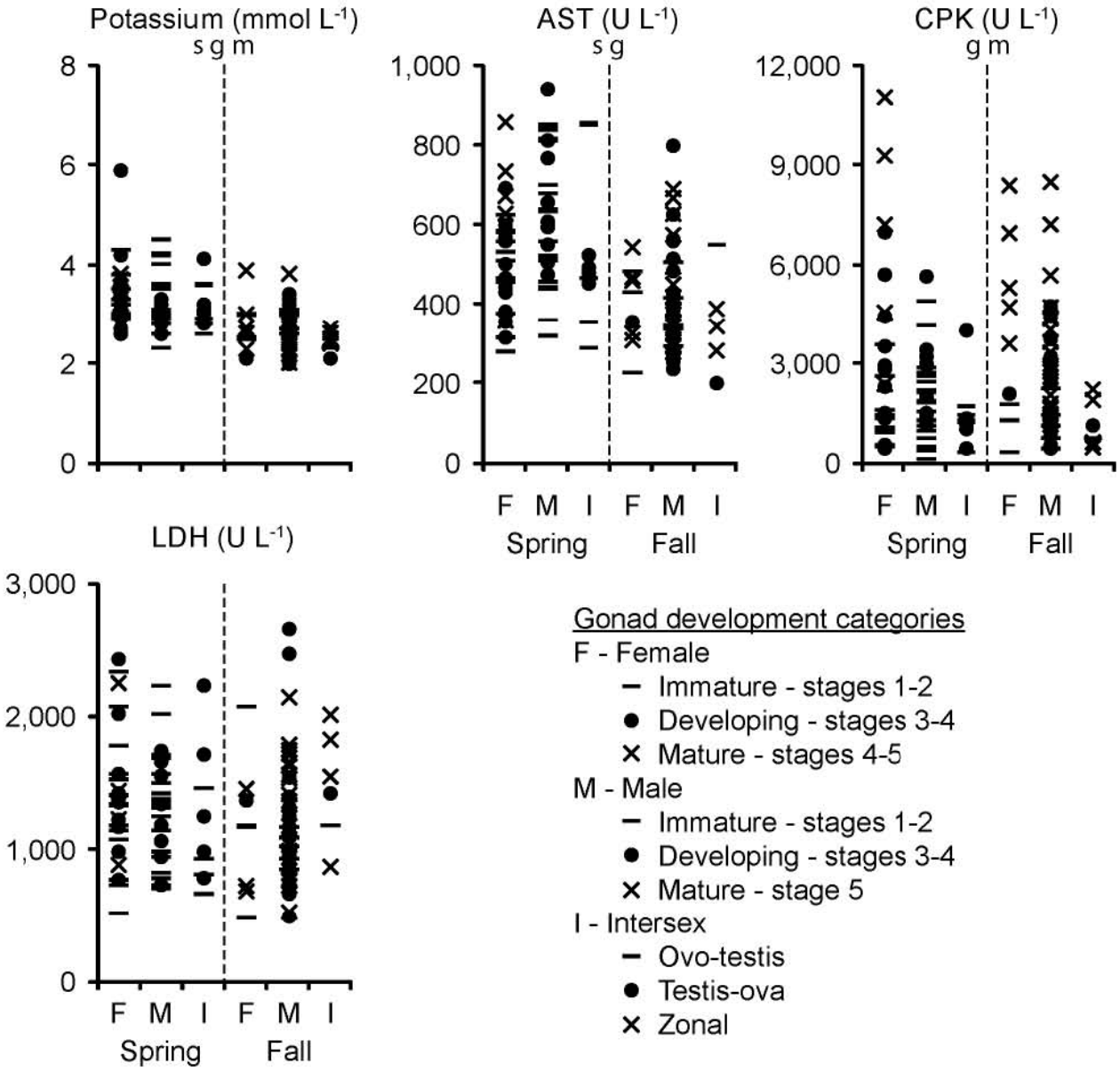

Total protein (g dl-1)
s m
Albumin (g dl-1)
s m
Globulin (g dl-1)
s m
Sodium (mmol L-1)
y s m
Chloride (mmol L-1)
y s m
Urea (mg dl-1)
s m
Glucose (mg dl-1)
g
Calcium (mg dl-1)
g m
Phosphorous (mg dl-1)
s g m
F M I F M I
Spring Fall

Fig. 2 continued



Chapter 3: Hematology and plasma chemistry of shortnose sturgeon during a dam-impeded spawning run

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Summary

Shortnose sturgeon (*Acipenser brevirostrum)* spawning migrations on the Cooper River are impeded by Pinapolous dam, lake Moultrie, South Carolina. Sturgeon and other species aggregate below the dam in winter/early spring and are subjected to a variety of stressors stemming from crowding, poor habitat quality and boat propeller and turbine damage. Spawning has been documented in the tailrace but reproductive success has not been verified as no juveniles have been captured. Fish within the dam tailrace were captured by gill net during winter, 2005 and 2007-2011, and physiological condition was assessed using a panel of hematologic and biochemical indices. Plasma phosphorous and PCV were significantly higher in males, while calcium and aspartate aminotransferase were significantly higher in females, indicating sex-specific physiological changes triggered during maturity. A marked leucopenia, accompanied by lymphopenia and neutrophilia, was evident in both sexes and was consistent across years indicating that these fish under chronic stress. Testosterone and estradiol levels were measured from these fish and hematologic and biochemical reference intervals were determined. Additional evaluations of shortnose sturgeon before or after spawning runs would be useful to determine the extent of physiological changes and potential recovery from stresses incurred during the winter spawn aggregation.

Introduction

Anecdotal reports of shortnose sturgeon in the Cooper River and the reservoirs above the river exist, but nothing was documented until 1995 when illegal gill nets containing the species were discovered in the tailrace of the Cooper River at Pinopolis Dam. Pinopolis Dam is the first impediment to upstream migration occurring at rkm 77. The dam was completed in 1941to provide hydroelectric power, navigation and flood control. The Dam produces power on a peaking basis and weekly average flows are maintained at 127 m^3/s. The navigation lock contains a 15 m vertical sill which allows for passage of boats and alosine fishes, but is not suitable for passage of bottom dwelling fish such as shortnose sturgeon. Because of this limitation to pass upstream to more suitable spawning habitat, shortnose sturgeon congregate at the base of the dam during late winter and early spring. Spawning has been verified at this site via fertilized egg collection and developing embryos (Cooke & Leach 2004, Duncan *et al.* 2004). It is unknown if viable recruitment occurs in the Cooper River because no juvenile fish have yet to be captured. Shortnose sturgeon captured in the Cooper River during 1996-1998 were tagged for a multiple census mark-recapture population estimate using the Schnabel method. The average population estimate indicated that there were only about 200 adult, spawning run shortnose sturgeon in the Cooper River (Cooke *et al.* 2004).

The site below Pinopolis Dam is atypical in terms of other published shortnose sturgeon spawning habitat in several ways, including distance upriver, tide, flow characteristics, and substrate. The river is tidally influenced for its entire length, and the bottom substrate in the tailrace is dominated by hard marl with deposits of Asian clam *Cobicula manilensis* shell and negligible amounts of silt and sand (Cooke & Leach 2004).

Hematologic and blood chemistry analysis can be a sensitive indicator of physiological condition and sublethal disease in animals. However the application of clinical pathology has been limited in fish because of a lack of standardized reference intervals for most species (Hrubec *et al.* 2000). While the majority of sturgeon research has focused on conservation biology and propagation, there is a need for non-lethal techniques to assess the physiological condition of imperiled fish such as sturgeon (see Webb & Doroshov 2011). In recent years, however, there has been increased efforts to establish "normal" hematologic and blood chemistry values in sturgeon (Bahmani *et al.* 2001, Asadi *et al.* 2006a, Asadi *et al.* 2006b, Knowles *et al.* 2006, Shi *et al.* 2006, Shahsavani *et al.* 2010a, Shahsavani *et al*. 2010b, DiMarco *et al*. 2011, Sadati *et al*. 2011), and to determine normal patterns of circulating hormone levels for use in determining sex or reproductive condition (Webb *et al*. 2002, Viayeh *et al*. 2006, Craig *et al*. 2009). A variety of studies have described physiological changes that occur in sturgeon under stress or different

environmental conditions (Baker *et al*. 2005, Jarvis *et al*. 2003, Kieffer *et al*. 2001, Potts & Rudy 1972, LeBreton *et al*. 1998, Barton *et al*. 2000). The goal of this study was to determine hematologic and plasma chemistry reference intervals, testosterone and estradiol levels, sex ratio and general health condition of mature shortnose sturgeon that aggregate in winter below Lake Moultrie dam, South Carolina. Annual variation and gender-specific differences in analyte values were evaluated.

Methods

Shortnose sturgeon were sampled from the Cooper River tailrace (rkm 77) just downstream from Pinopolis Dam. Fish were collected using 100 m experimental multi-filament gillnets. In 2005 and 2007-2010, gillnets consisted of six, 15.2 m panels with alternating stretch mesh sizes of 125, 175 and 255 mm. During 2011, the gillnets consisted of only 175 mm stretched mesh. Nets were set approximately ten minutes after turbine generation subsided at Pinopolis Dam and were allowed to soak for fifteen minutes before being checked.

Up to 12 shortnose sturgeon were captured annually in February or March, in 2005 and 2007-2011. Captured fish were transported in river water and placed in a float pen next to an on-shore location where fish assessments were performed. Once on-shore, fish were examined, weighed, measured, scanned for passive integrated transponder (PIT) tags, and a tissue sample from the pectoral fin was taken for genetics. Most of the fish captured were either gravid females or running ripe males. When the gender of a fish was not obvious, sex was determined by visually assessing the gonads using the laparoscopic techniques of Matsche *et al*. (2011). A 5-ml blood sample was collected from the caudal vein of each fish and transferred to blood collection tubes containing lithium heparin and plasma separator gel (Vacutainer® and Microtainer®, BD, Franklin Lakes, NJ). Total white blood cell count (WBC), PCV and leukograms were determined according to Knowles *et al.* (2006). Remaining blood was centrifuged at 10,000 x g for 5 min and plasma was decanted and chilled for storage. Glucose, urea, total protein, albumin, aspartate aminotransferase (AST), calcium, phosphorous, sodium, potassium, chloride, globulin, creatinine phosphokinase (CPK) and lactate dehydrogenase (LDH) were measured using an automated chemistry system (AU-5400, Olympus America Inc., Center Valley, PA). Plasma for hormone analysis was frozen in a liquid nitrogen dewar and shipped on dry ice to the Cooperative Oxford Laboratory for analysis.

Testosterone and estradiol were measured from plasma using commercially available ELISA assay kits (Cayman Chemical, Ann Arbor, MI) according to manufacturer's instructions. All samples were extracted in diethyl

ether; the cold spike method was used to determine extraction efficiency and samples were analyzed with a uQuant spectrophotometer (Biotek Instruments, Inc., Winooski, VT) at a wavelength of 410 nm. Average extraction efficiencies were 77.2% for testosterone and 86.3% for estradiol. The reported detection limits of the ELISA kits at %B/Bo of 80% are 6 pg ml^{-1} for testosterone and 19 pg ml^{-1} for estradiol. The reported cross-reactivities to other similar antibodies is <5% for testosterone antibodies and ≤1% for estradiol antibodies (Cayman Chemical). The intra-assay variation of the ELISA kits was 6.9% for testosterone and 5.6% for estradiol. The inter-assay variation was 13.4% for testosterone and 10.3% for estradiol.

Fish weight, TL, hematologic and plasma chemistry analyte data were tested for normal distribution (Kolmogorov-Smirnoff) and homoschedasticity (Bartlett). One-way analysis of variance (ANOVA) with general linear model was used to test the effects of year and gender on weight, TL, testosterone and estradiol. Transformed and untransformed hematologic and plasma chemistry data failed to meet the assumptions required for parametric analysis. Therefore, nonparametric ANOVA (Kruskal-Wallis), using sequential Bonferroni correction (Rice 1989)) was used to evaluate the effect of year and gender on hematology and plasma chemistry data. When a significant gender effect was detected, hematologic and plasma chemistry reference intervals were determined for males and females separately using robust procedures (Horn *et al*. 1998) according to guidelines approved by the Clinical Laboratory Standards Institute (CLSI 2008). For all remaining analytes, a single reference interval was determined from combined data. All statistical analysis was performed using SAS Enterprise Guide 4.1 (Davis 2007)

Results

Dissolved oxygen was > 8 ppt and temperature varied from 8-13 in this study (Table 1). Hematologic and blood chemistry analysis was performed on a total of 71 adult, mature fish (Table 2). Females were significantly larger in TL and weight than males (Table 3). Sex was determined by laparoscopy on 26 of the 71 fish. All fish survived procedures and were released to the Cooper River within minutes following procedures. Testes from all males were enlarged, white and had no outer layer of fat indicating stage 5 (see Matsche *et al*. 2011). Ovaries of all females were enlarged with black mature oocytes indicating stage 6 (see Matsche *et al*. 2011). During this study several fish were recaptured in subsequent years after procedures were performed. Incisions in the ventral skin (for cannulae installation) from laparoscopic examination the previous year were either not evident or appeared as a faint, pigmented line (Fig. 2). The majority of the fish were robust and in good condition, but some fish had deformities

or injuries prior to being captured, including skin lacerations across the dorsal body surface (Fig. 3) and damaged or missing rostrum (fig. 4) or fins (Fig. 5). Intact skin and lack of inflammation indicated that at least some of the traumatic injuries did not occur recently. However, several skin lacerations and the caudal peduncle of a fish with a missing caudal fin were open, bleeding or reddened, indicating recent trauma. Overall sex ratio of sampled fish was not significantly different from an expected 1:1 ratio (Table 2).

Blood cells evident from fish in this study were morphologically similar to those described from juvenile shortnose sturgeon (Knowles *et al*. 2006). Erythrocytes (≈ 17 x 10 μm) were oval with a smooth, eosinophilic cytoplasm and centrally located nucleus (Fig. 6). Thrombocytes (≈ 15 x 6 μm) had a faint, pink cytoplasm and were spindle, pyriform or oval in shape (Fig. 6). Small lymphocytes (≈ 8 x 6 μm) and large lymphocytes (≈ 12 x 10 μm) had a narrow rim of blue cytoplasm with extensive cytoplasmic pseudopodia and a large, round nucleus (Fig. 6). Monocytes (≈ 17 x 13 μm) were generally larger than large lymphocytes, contained a wider rim of blue cytoplasm and were usually vacuolated (Fig. 6). Neutrophils (≈ 17 x 15 μm) were highly irregular in shape with a faint blue cytoplasm that had a grainy texture, and a reniform or segmented nucleus (Fig. 6). Eosinophils (≈ 17 x 15 μm) were round to oval with numerous pink eosinophilic granules and a reniform or segmented nucleus (Fig. 6).

Annual variation in hematologic and plasma chemistry data was low (Table 4, Figs. 7-8). Year had a significant effect only on eosinophil counts (Table 4). Median PCV was significantly higher in males than in females and was therefore partitioned into gender-specific reference intervals (Table 5, Fig. 9). Total WBC counts were low, neutrophils were the most abundant leukocyte and reference interval of N:L ratios were 1.05-2.29 (Table 5). Calcium and AST were significantly higher in females while phosphorous was significantly higher in males; reference intervals for those analytes were partitioned by gender (Table 6, Fig. 10).

There was a significant effect of gender on hormone concentrations (Table 7). Annual variation in hormone levels was relatively small; no effect of year was detected (Table 7, Fig. 11). Hormone levels by gender are presented in Fig. 12.

Discussion

Upstream migration of the Cooper River population of shortnose sturgeon is severely limited at Pinopolis Dam. Shortnose sturgeon congregating and spawning in the tailrace of the dam has been documented but no viable fry or juvenile fish have been captured. The population may be depressed to an unsustainable level as a result of poor

recruitment that is linked to obstructed migration to favorable spawning habitat (Cooke *et al*. 2004). After spawning season, adult shortnose sturgeon migrate back downriver between rkm 22-48 where they spend the remainder of the year (Palmer 2001).

Besides the fact these fish are being forced to spawn below the dam because of inadequate upstream passage, another potential stressor to the population may be overcrowding. Shortnose sturgeon are congregated below the dam during late winter through early spring when alosine spawning runs are taking place. Large schools of American shad, *Alosa Sapidissima*, blueback herring, *Alosa aestivalis*, and predatory fish such as blue catfish, *Ictalurus furcatus*, and striped bass, *Morone saxatilis*, are all congregated at the base of the dam competing for space.

A wide variety of factors including environmental (e.g. temperature, seasonality, salinity and oxygen levels) and biotic (e.g. age, sex, reproductive state, genetic variation and activity levels) can influence blood values in fishes (Hrubec & Smith 2000). Furthermore, differences in hematologic techniques and analysis can produce variable results (DiMarco *et al*. 1999). Therefore reference intervals partitioned by gender, reproductive status, season, habitat (e.g. cultured vs wild, salinity) or other factors, may be particularly important for species such as sturgeon that are long-lived, have prolonged reproductive cycles and can undergo extensive migrations. Also, blood values can differ significantly among sturgeon species (Shi *et al*. 2006), highlighting the need for species-specific intervals. Prior to this study, the only available reference intervals for shortnose sturgeon were determined for cultured, subadult fish of unknown sex (Knowles *et al*. 2006), potentially limiting their applicability to wild or mature fish. Studies that documented hematologic responses to salinity acclimation and exercise in shortnose sturgeon (Kieffer *et al*. 2001, Jarvis & Ballantyne 2003, Baker *et al*. 2005, Beya *et al*. 2005) offer insight into stress physiology, but are of limited diagnostic usefulness because sample sizes were small, few analytes were evaluated and reference intervals were not determined.

In addition to the study of Knowles *et al*. (2006), "normal" blood values, intended as reference values, have been reported from *A. stellatus* (Shahsavani *et al*. 2010a, Shahsavani *et al*. 2010b), *A. schrenckii* and *A. sinensis* (Shi *et al*. 2006), *A. persicus* (Bahmani *et al*. 2001, Asadi *et al*. 2006a), *Huso huso* (Bahmani *et al*. 2001, Asadi *et al*. 2006b), *A. baerii* (Sadati *et al*. 2011) and cultured sturgeon hybrids (*A. naccarii* female × *A. baerii* male, DiMarco 2011). With the exception of Knowles *et al*. (2006) and DiMarco *et al*. (2011), these studies report only descriptive summaries of the data (mean and standard deviations) rather than reference intervals, and relied on very small

sample sizes, ranging from 6-20 fish (Asadi *et al*. 2006a, Asadi *et al*. 2006b, Shi *et al*. 2006, Shahsavani *et al*. 2010a, Shahsavani *et al*. 2010b, Sadati *et al*., 2011). Knowles *et al*. (2006) and DiMarco *et al*. (2011) reported nonparametric reference intervals for shortnose sturgeon and sturgeon hybrids, but the sample sizes used in those studies (46-77 and 60 respectively) fell short of the recommended number of animals (120) for nonparametric techniques (CLSI 2008). Achieving sufficient sample sizes for reference interval determinations is often problematic, particularly with endangered or imperiled species such as sturgeon. To address this issue, robust techniques were developed as an improved method of determining reference intervals from small or skewed datasets (Horn *et al*. 1998). The use of excessively small sample sizes in reference studies ($n \leq 20$) increases the risk of large errors in the resulting estimates, resulting in reference intervals that do not reflect the true underlying distributions in the populations sampled, and may overestimate the upper endpoint of the interval (Horn *et al*. 1998). Robust techniques are more tolerant of outliers, do not require transformation and can more accurately predict estimates with limited or skewed data (Horn 1988, Horn *et al*. 1998).

Plasma calcium was significantly higher in females in this study. A fraction of total calcium is bound by proteins in the plasma of fish, and VTG is a major calcium binding protein (Mcdonald & Milligan 1992). As VTG increases with maturity in females, there is a proportional increases in plasma calcium (Linares-Casenave *et al.* 2003). In contrast, plasma calcium did not differ between male and female starry sturgeon, but reproductive stage of fish examined was not reported and sample size was small (Shahsavani *et al*. 2010b). It is possible that previtellogenic females were included in the study by Shahsavani *et al*. (2010b), masking potential effects of gender on calcium. Because plasma calcium levels are directly related to reproductive stage in sturgeon, studies documenting calcium reference intervals should clearly define the reproductive stages of fish used or partition reference intervals by maturity stage when possible (Webb *et al.* 2002, Linares-Casenave *et al.* 2003).

Plasma AST also differed significantly by gender in this study. Increased AST activities in fish could indicate general stress, infection, impaired liver or kidney function or increased metabolism of amino acids (Evenberg *et al*. 1986, Wagner & Congleton 2004, Nie *et al*. 2007, Feng *et al*. 2011). However, it is more likely that differences exhibited in AST by gender indicate natural variation in enzyme activities during the reproductive cycle. Significantly higher AST activity was also reported in mature female *A. stellatus* than in males (Shahsavani *et al*. 2010a). In *A. persicus*, AST activities were significantly higher in mature females than in immature females and

males (Asadi *et al*. 2006a). Aspartate is an important energy substrate in fish (Li *et al*. 2009) and increased AST in females during maturity may reflect increased energetic demands of egg production.

Plasma phosphorous in males were higher than in females in mature shortnose sturgeon (this study) and juvenile *H. huso* (Asadi *et al*. 2006b). In contrast, phosphorous was higher in adult female *A. stellatus* than in males (Shahsavani *et al*. 2010b). Levels of phosphorous reported here for mature shortnose sturgeon are similar to those reported in Atlantic sturgeon (Baker *et al*. 2005) and juvenile *H. huso* (Asadi *et al*. 2006b), mature *A. stellatus* (Shahsavani *et al*. 2010b) and juvenile hybrid sturgeon (DiMarco *et al*. 2011). However the reference intervals reported here for male and female mature shortnose sturgeon are higher than that of juvenile, cultured shortnose sturgeon (5.1 to 8.1, Knowles *et al*. 2006).

PCV is a widely used indicator of health condition in fish and typically ranges from 20 to 45%, with higher values more commonly found in active species and lower values more common in sedentary fish (Hrubec & Smith 2000). A wide variety of variables can alter PCV including age, sex, reproductive state, temperature, salinity and season (Hrubec & Smith 2000). Decreased PCV can occur with disease, parasitism, poor nutrition, exposure to toxicants and hemorrhage secondary to other causes such as trauma and disease (Clauss *et al*. 2008). Increased PCV can occur in mature males (Blaxhall 1972), in response to hypoxia (Fänge 1992), dehydration (Campbell 2004) and stressors that trigger erythrocyte swelling or mobilization (McDonald & Milligan 1992).

The marked leukopenia evident in this study (3,424-6,261 total WBC) is a general indicator of stress in fish (Noga 2000). Reported normal leukocyte counts in sturgeon are typically much higher (Knowles *et al.* 2006, Zarejabad *et al*. 2010). Mean total WBC ranged from 18,200 to 22,300 in *H. huso*, depending on salinity (Zarejabad *et al*. 2010), from 19,405 to 25,480 in *A. baeri*, depending on temperature (Sadati *et al*. 2011), and reference interval reported for cultured, juvenile shortnose sturgeon was 28,376 to 90,789 (Knowles *et al*. 2006). Handling and confinement stress, exposure to a variety of toxicants and disease can result in a decrease in total leukocytes in fish, often with a concomitant lymphopenia and neutrophilia, although the response can vary by fish species and stressor (Noga 2000). Increased N:L ratios are often used as a measure of stress (Feldman *et al*. 2000), and in sturgeon are usually < 0.6 (Knowles *et al*. 2006, Zarejabad *et al*. 2010). However, shortnose sturgeon sampled in this study had severely depleted lymphocyte counts (1,141 to 2,144 total) and low neutrophil counts (1,533 to 4,085) in comparison to other studies (Knowles *et al*. 2006, Zarejabad *et al*. 2010), resulting in elevated N:L ratios in this study (1.05 to 2.29). It is also possible that low wintertime temperatures contributed to the drastic hematologic

response evident in this study (Hrubec *et al* 1997). A consequence of severe leucopenia could be impaired immune function and increased susceptibility to disease (Feldman *et al*. 2000).

Hormone levels reported in this study were higher than in mature shortnose sturgeon from Delaware River (see Chapter 1). In males, mean testosterone was 38.1 ng ml^{-1} in Cooper River fish, and was 26 ng ml^{-1} in Delaware River fish. In females, estradiol was 2.7 ng ml^{-1} in Cooper River fish, and was 0.9 ng ml^{-1} in Delaware River fish. However, the majority of fish in this study were running ripe males or gravid females, while mature fish examined from the Delaware River were either pre-ovulatory females or pre-spermiating males. Testosterone and estradiol levels often decrease during final maturation in sturgeon and other fishes (Kime 1993, Semenkova *et al.* 2002, Barannikova *et al.* 2004). Testosterone is related to spermatogenenesis, and often decreases during final maturation as steroidogenic activity shifts towards progestin synthesis (Yaron 1995). Estradiol is positively correlated to vitellogenin synthesis, and decreases upon completion of vitellogenesis (Kime 1993). In sturgeon there is a high degree of variability in hormone profiles within and among species (Barannikova *et al.* 2004, Davail-Cuisset *et al.* 2011). Therefore differences in hormone profiles between population segments that are genetically distinct and separated latitudinally and are not unexpected.

Hematologic and chemistry values reported here differ considerably than “normal” values reported in other sturgeon species. This is not entirely surprising as fish exhibit high variability in blood values as a consequence of the diverse environmental conditions they occupy and physiological adaptations. This high degree of diversity complicates interpretation of and diagnostic usefulness of blood value results in fish, and highlights the need for well-defined, standardized reference intervals partitioned by life stage, sex, maturity or other factors. Therefore more work is needed to further partition reference intervals for shortnose sturgeon and other species.

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Tables and Figures

Table 1
Water quality from Cooper River.

Year	DO (ppt)	Temperature (°C)
2005	11.7-11.8	8.3-9.5
2007	9.7-12.4	8.8-9.3
2008	9.9-10.9	11.5-12.2
2009	10.4-10.8	9.7-9.8
2010	12.5	7.3-8.2
2011	8.9-10.3	12.5-13.2

Table 2
Size weight and sex ratio of shortnose sturgeon collected from Cooper River, South Carolina, 2005-2011.

	Male			Female			Sex ratio	
Year	n	TL (mm)	Weight (kg)	n	TL (mm)	Weight (kg)	M:F	Fisher's Exact P
2005	5	956 ± 13	5.4 ± 0.2	7	1039 ± 20	9.5 ± 0.9	0.7:1	1.0000
2007	8	951 ± 15	5.9 ± 0.5	4	1054 ± 54	10.1 ± 2.0	2:1	0.6802
2008	5	892 ± 20	4.8 ± 0.6	7	1112 ± 21	11.6 ± 0.6	0.7:1	1.0000
2009	6	957 ± 28	5.2 ± 0.3	5	1110 ± 39	11.2 ± 1.1	1.2:1	1.0000
2010	4	904 ± 17	5.1 ± 0.3	8	1119 ± 40	12.2 ± 0.9	0.5:1	0.6802
2011	10	937 ± 30	5.0 ± 0.4	2	1120 ± 44	11.5 ± 2.4	5:1	0.1930
Overall	38	935 ± 11	5.3 ± 0.2	33	1092 ± 14	11.1 ± 0.4	1.2:1	0.7372

Table 3
Comparison (ANOVA) of TL and weight among years and gender.

	Source	DF	F value	P
TL	Sex	1	68.89	<0.0001
	Year	5	2.03	0.0868
	Sex x year	5	1.44	0.2240
Weight	Sex	1	128.17	<0.0001
	Year	5	0.45	0.0002
	Sex x year	5	1.20	0.3221

Table 4
Annual variation of plasma chemistry and hematology data collected from shortnose sturgeon in winter, 2005-2011 from Cooper River, South Carolina. Coefficient of variation (CV) was determined by bootstrap technique (n=1000). Year of sampling had no effect on any analytes.

				Kruskal-Wallis	
Analyte	n	CV	d.f.	Chi-square	P
Total protein	72	0.1824	5	0.8439	0.9741
Albumin	72	0.1876	5	9.0175	0.0785
Glucose	72	0.3918	5	7.6064	0.1793
Urea nitrogen	72	0.5379	5	3.2630	0.6595
Calcium	72	0.3467	5	8.7375	0.1808
Phosphorus	72	0.2106	5	5.7778	0.3284
Sodium	72	0.0442	5	8.9791	0.0826
Potassium	72	0.2889	5	8.2543	0.1429
Chloride	72	0.0479	5	9.0080	0.1027
Globulin	72	0.2319	5	4.7923	0.4418
AST[1]	72	0.4466	5	1.7078	0.8879
CPK[2]	72	0.8981	5	2.8262	0.7268
LDH[3]	72	0.4621	5	2.0150	0.8471
PCV	71	0.2222	5	9.4748	0.0916
Total WBCs	71	0.5284	5	4.7487	0.4473
Small lymphocytes	71	0.4481	5	5.2878	0.3618
Large lymphocytes	71	0.6471	5	7.3386	0.1818
Neutrophils	71	0.6857	5	8.0865	0.1515
Monocytes	71	1.9825	5	4.7185	0.4512
Eosinophils	71	2.7963	5	10.7762	0.0118
Neutrophil:lymphocyte ratio	71	0.7888	5	9.1114	0.0621

[1] aspertate aminotransferase
[2] creatinine phosphokinase
[3] lactate dehydrogenase

Table 5
Hematology reference intervals for adult shortnose sturgeon collected from the Cooper River, South Carolina in winter, 2005-2011 (n=72). For each analyte, P values followed by an asterisk (*) indicate a significant effect of gender. Separate robust reference intervals are provided for analytes with a significant gender effect.

									Robust Interval	
Analyte	Sex	n	Median	25th percentile	75th percentile	Kruskal-Wallis Chi-square	d.f.	P	Lower limit	Upper limit
PCV (%)	M	38	41	34	46	30.2054	1	<0.0001*	39	44
	F	33	30	27	34				29	32
Total WBCs (cells μL^{-1})	M	38	4,750	3,000	7,000	0.2775	1	0.5983	3,424	6,261
	F	33	5,000	3,000	7,000					
Small lymphocytes (cells μL^{-1})	M	38	1,220	660	1,750	1.4223	1	0.2330	826	1,969
	F	33	1,680	960	2,160					
Large Lymphocytes (cells μL^{-1})	M	38	720	220	1,175	0.2511	1	0.6789	315	1,075
	F	33	780	260	1,310					
Neutrophils (cells μL^{-1})	M	38	2,628	1,620	4,400	0.0176	1	0.8946	1,533	4,085
	F	33	3,276	1,280	4,740					
Monocytes (cells μL^{-1})	M	38	80	0	280	0.7378	1	0.3904	0	355
	F	33	140	0	360					
Eosinophils (cells μL^{-1})	M	38	0	0	30	0.1120	1	0.7379	0	48
	F	33	0	0	60					
N:L ratio	M	38	1.35	0.58	2.12	1.0546	1	0.3045	1.05	2.29
	F	33	1.33	0.68	2.43					

Table 6
Plasma chemistry intervals for adult shortnose sturgeon collected from the Cooper River, South Carolina in winter, 2005-2011 (n=72). For each analyte, P values followed by an asterisk (*) indicate a significant effect of gender. Separate robust reference intervals are provided for analytes with a significant gender effect.

									Robust Interval	
Analyte	Sex	n	Median	25th percentile	75th percentile	Kruskal-Wallis Chi-square	d.f.	P	Lower limit	Upper limit
Total protein (g dl^{-1})	M	38	5.4	4.7	5.8	1.0307	1	0.3100	2.9	3.3
	F	33	5.2	4.4	5.7					
Albumin (g dl^{-1})	M	38	1.9	1.7	2.1	6.4157	1	0.0113	1.8	2.2
	F	33	2.1	1.8	2.4					
Glucose (mg dl^{-1})	M	38	76	55	99	1.2575	1	0.2621	61	80
	F	33	69	58	90					
Urea nitrogen (mg dl^{-1})	M	38	2	2	3	5.2294	1	0.0222	1.5	2.5
	F	33	2	1	2					
Calcium (mg dl^{-1})	M	38	10.4	9.9	10.8	47.5761	1	<0.0001*	10.2	10.9
	F	33	19.2	15.6	22.1				18.3	20.7
Phosphorus (mg dl^{-1})	M	38	13.3	12.9	14.7	8.9746	1	0.0027*	12.6	14
	F	33	12.1	10.1	14.4				10.9	13.3
Sodium (mEq L^{-1})	M	38	153	151	158	2.6476	1	0.1037	148	155
	F	33	151	149	155					
Potassium (mEq L^{-1})	M	38	1.7	1.6	2.6	5.3657	1	0.0205	1.4	2.6
	F	33	2.2	1.8	2.8					
Chloride (mEq L^{-1})	M	38	125	122	129	3.3852	1	0.0658	123	131
	F	33	129	124	132					
Globulin (g dl^{-1})	M	38	3.6	3.0	3.9	5.9401	1	0.0148	2.7	3.8
	F	33	3.0	2.6	3.7					

Table 6 continued

AST[1] (U L^{-1})	M	38	452	317	563	26.8667	1	<0.0001*	179	214
	F	33	253	191	301				223	289
CPK[2] (U L^{-1})	M	38	1,824	1,083	2,149	1.1587	1	0.2817	1,419	2,730
	F	33	1,999	923	3,817					
LDH[3] (U L^{-1})	M	38	879	710	1,052	2.0546	1	0.3045	733	980
	F	33	765	633	794					

[1] aspertate aminotransferase
[2] creatinine phosphokinase
[3] lactate dehydrogenase

Table 7
Comparison (ANOVA) of testosterone and estradiol among years and gender.

Analyte	Source	DF	F value	P
Testosterone	Sex	1	117.8	<0.0001
	Year	5	0.84	0.2871
	Sex x year	5	3.91	0.1188
Estradiol	Sex	1	189.68	<0.0001
	Year	5	0.68	0.2292
	Sex x year	5	0.79	0.4447

Figure captions

Fig. 1. Shortnose sturgeon capture site on the Cooper River, South Carolina, 2005-2011.

Fig. 2. Ventral skin of shortnose sturgeon 1 year after laparoscopic examination. Arrow indicates incision.

Fig. 3. Skin laceration on shortnose sturgeon from Cooper River, South Carolina.

Fig. 4. Shortnose sturgeon with missing rostrum, Cooper River, South Carolina.

Fig. 5. Shortnose sturgeon with missing caudal fin, Cooper River, South Carolina.

Fig. 6. Blood cells typical of shortnose sturgeon from Cooper River, South Carolina, winter 2005-2011 (a-d). E, erythrocytes; M, monocytes; LL, large lymphocytes; SL, small lymphocytes; N, neutrophils; E, eosinophils; T, thrombocytes. Scale bar = 20 µm

Fig. 7. Annual hematology of shortnose sturgeon from Cooper River, South Carolina, 2005-2011. Boxes indicate 75^{th} percentile (top line), median (middle line) and 25^{th} percentile (bottom line) of data range, while error bars indicate extreme values.

Fig. 8. Annual plasma chemistry of shortnose sturgeon from Cooper River, South Carolina, 2005-2011. Boxes indicate 75^{th} percentile (top line), median (middle line) and 25^{th} percentile (bottom line) of data range, while error bars indicate extreme values.

Fig. 9. Hematology by gender in shortnose sturgeon from Cooper River, South Carolina, 2005-2011. Bars with different letters indicate significant difference.

Fig. 10. Plasma chemistry by gender in shortnose sturgeon from Cooper River, South Carolina, 2005-2011. Bars with a different letter indicate significant difference.

Fig. 11. Annual hormone levels in shortnose sturgeon from Cooper River, South Carolina, 2005-2011. Boxes indicate 75th percentile (top line), median (middle line) and 25th percentile (bottom line) of data range, while error bars indicate extreme values.

Fig. 12. Hormone levels by gender in shortnose sturgeon from Cooper River, South Carolina, 2005-2011. For each hormone, bars with a different letter indicate significant difference.

Fig. 1

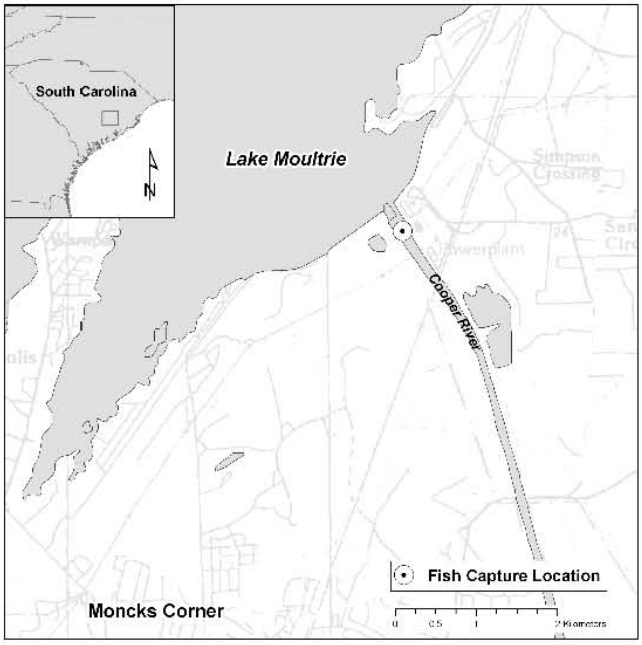

South Carolina
N
Lake Moultrie
Cooper River
Fish Capture Location
Moncks Corner

Fig. 2

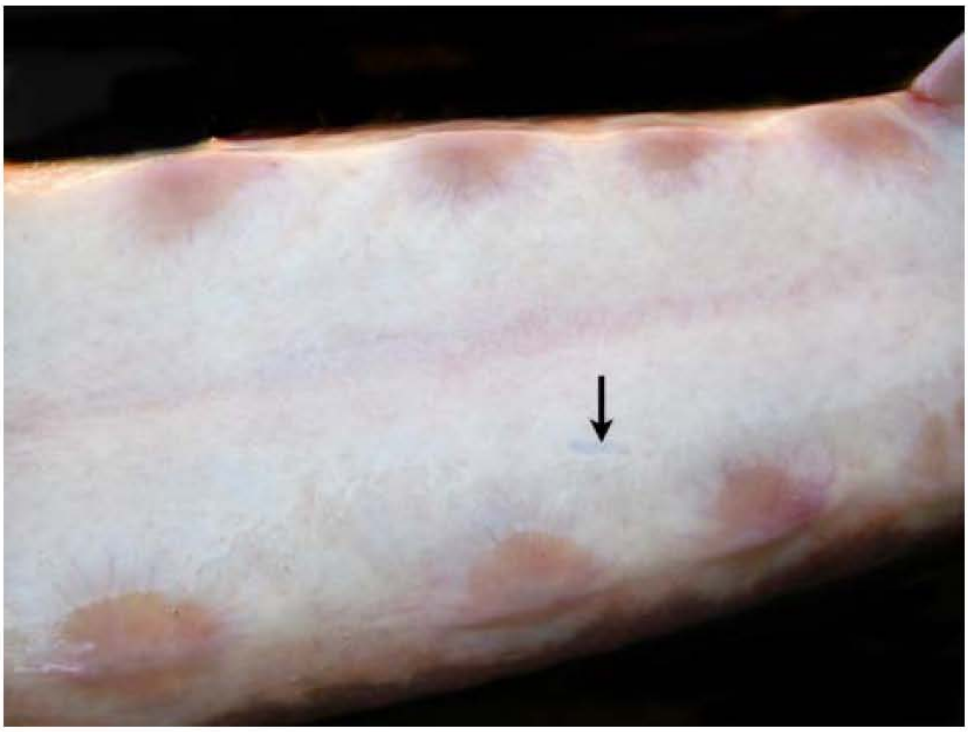


Fig. 3

Fig. 4

Fig. 5

Fig. 6

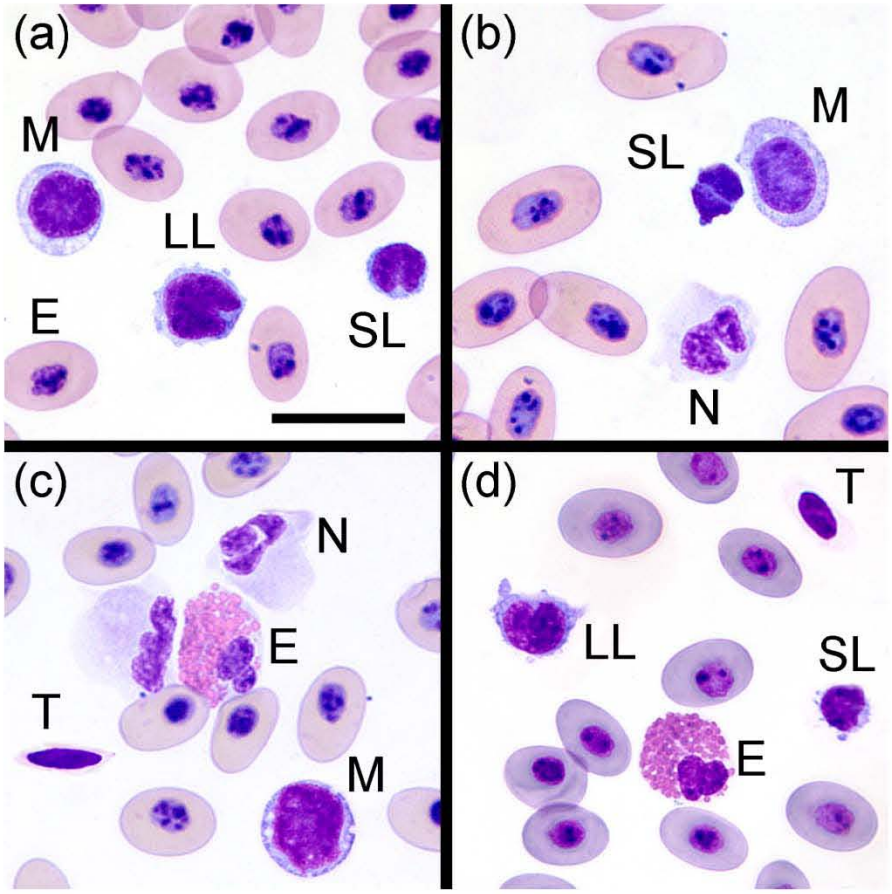


Fig. 7

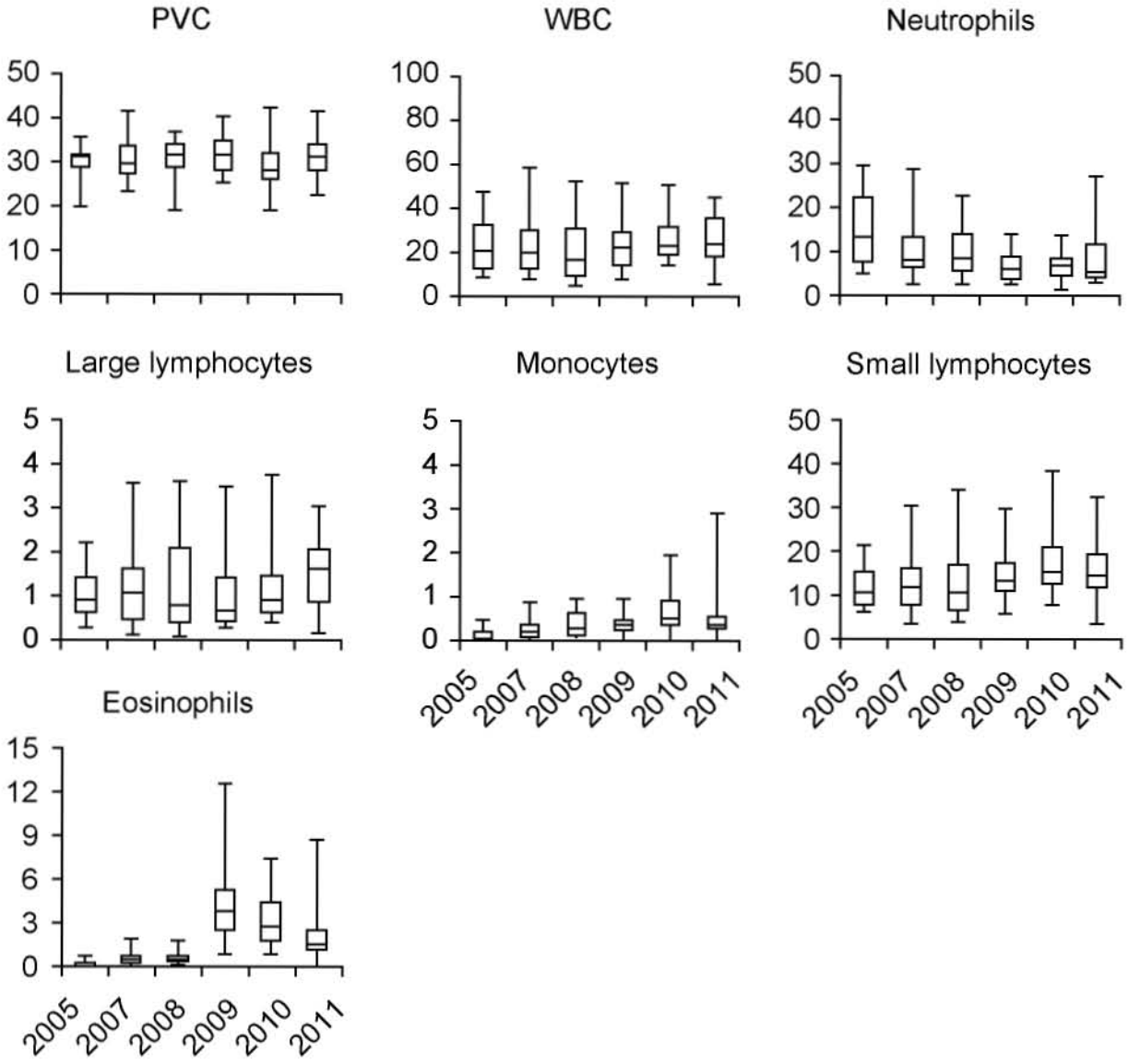

PVC
WBC
Neutrophils
Large lymphocytes
Monocytes
Small lymphocytes
Eosinophils
2005
2007
2008
2009
2010
2011

Fig. 8

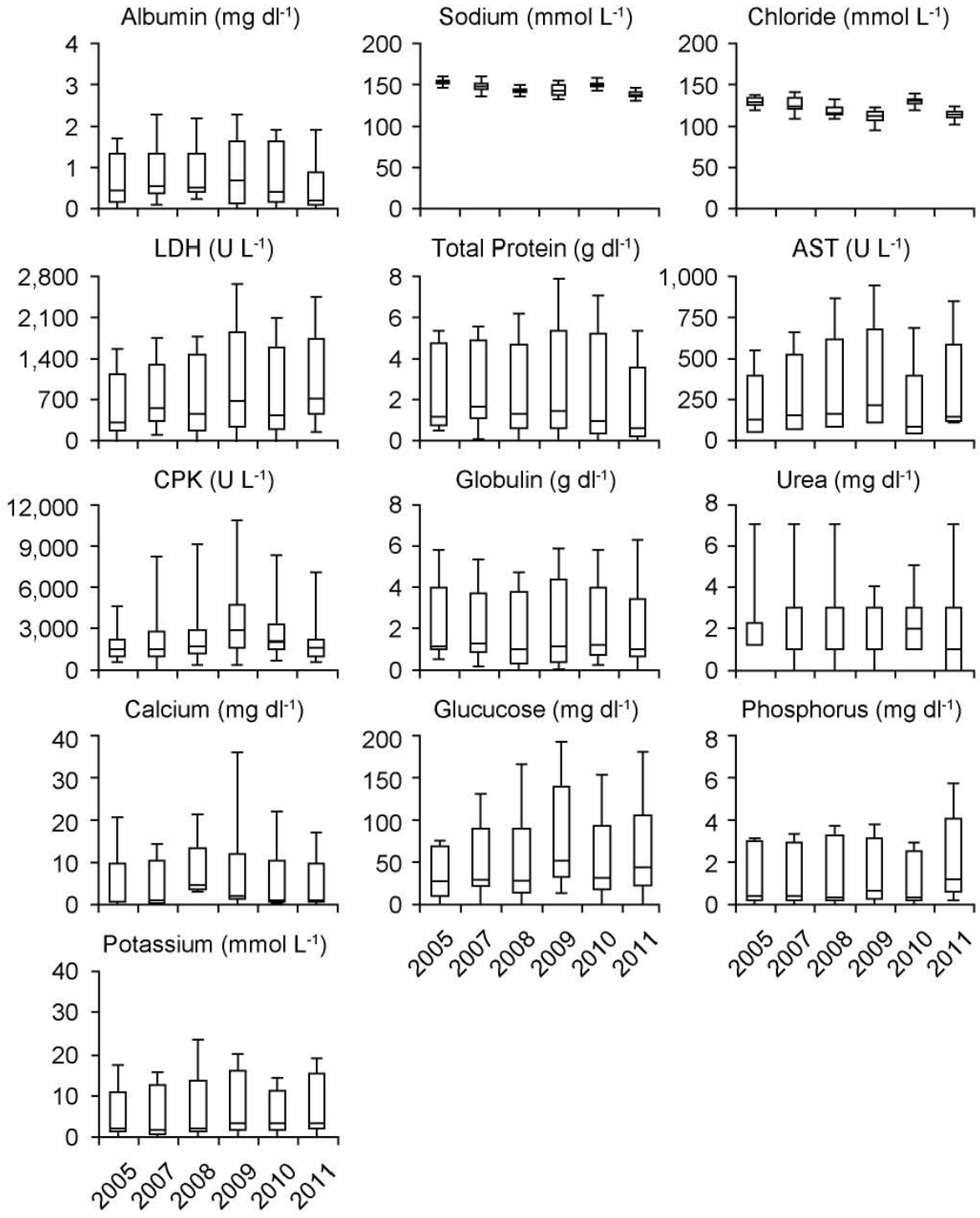

Albumin (mg dl-1)
Sodium (mmol L-1)
Chloride (mmol L-1)
LDH (U L-1)
Total Protein (g dl-1)
AST (U L-1)
CPK (U L-1)
Globulin (g dl-1)
Urea (mg dl-1)
Calcium (mg dl-1)
Glucucose (mg dl-1)
Phosphorus (mg dl-1)
Potassium (mmol L-1)
2005
2007
2008
2009
2010
2011

Fig. 9

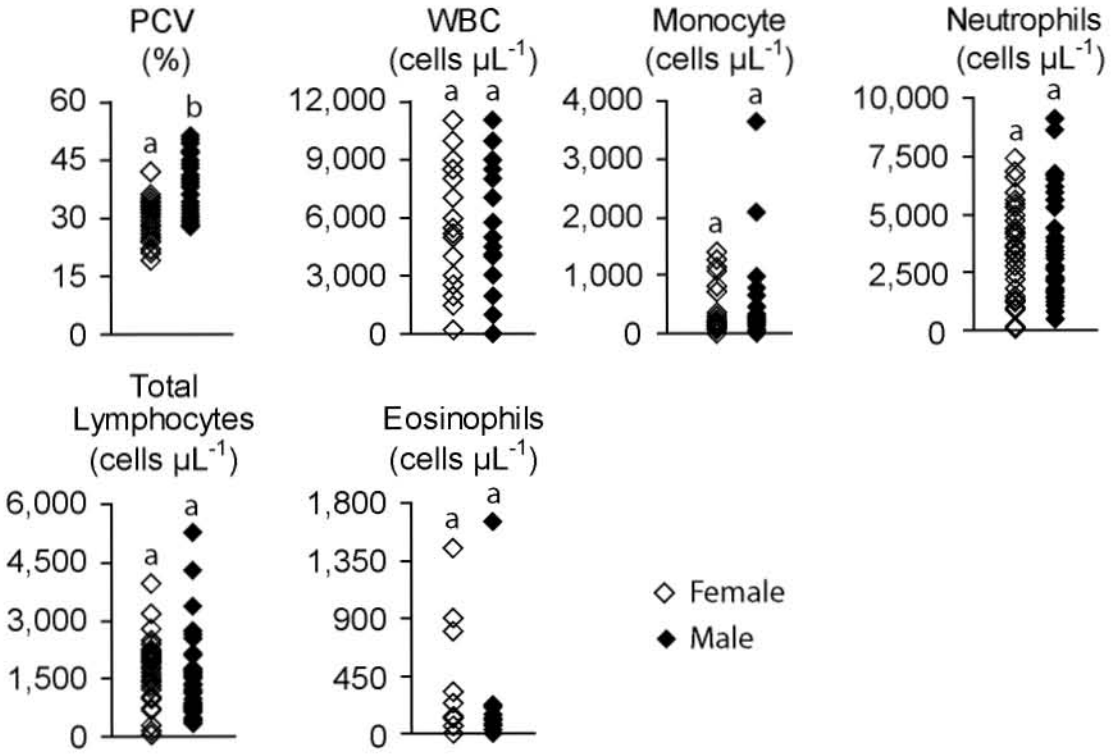

PCV (%)
60
45
30
15
0
a
b
WBC (cells µL-1)
12,000
9,000
6,000
3,000
0
a
a
Monocyte (cells µL-1)
4,000
3,000
2,000
1,000
0
a
a
Neutrophils (cells µL-1)
10,000
7,500
5,000
2,500
0
a
a
Total Lymphocytes (cells µL-1)
6,000
4,500
3,000
1,500
0
a
a
Eosinophils (cells µL-1)
1,800
1,350
900
450
0
a
a
Female
Male

Fig. 10

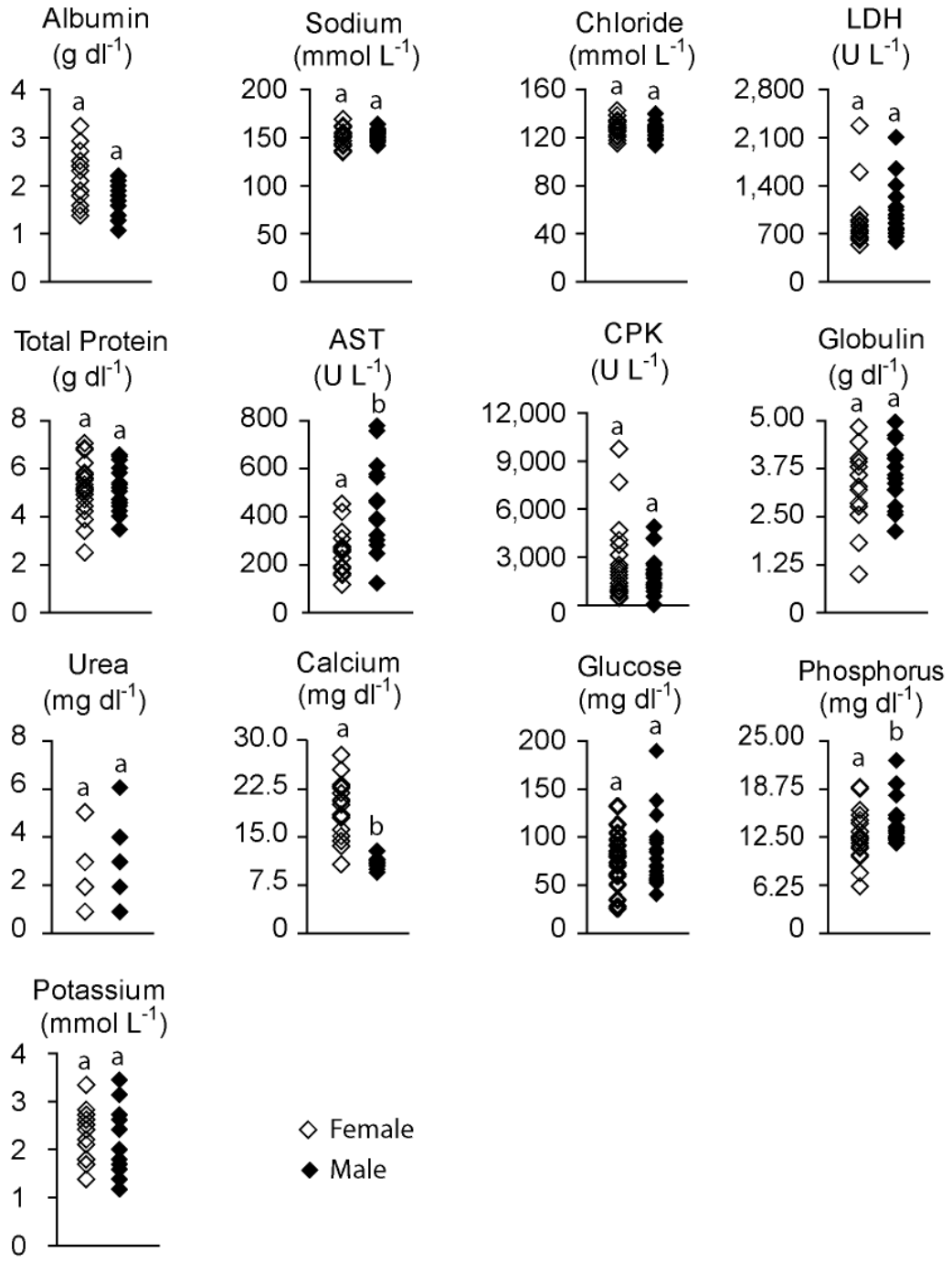

Albumin
(g dl-1)
Sodium
(mmol L-1)
Chloride
(mmol L-1)
LDH
(U L-1)
Total Protein
(g dl-1)
AST
(U L-1)
CPK
(U L-1)
Globulin
(g dl-1)
Urea
(mg dl-1)
Calcium
(mg dl-1)
Glucose
(mg dl-1)
Phosphorus
(mg dl-1)
Potassium
(mmol L-1)
Female
Male

Fig. 11

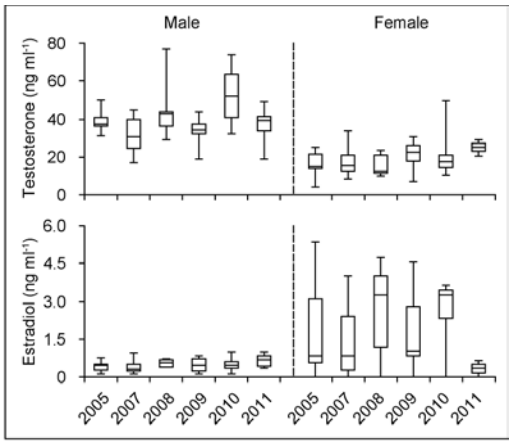
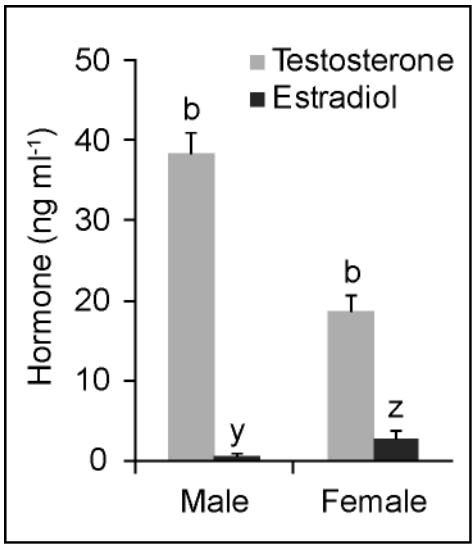

Male
Female
Testosterone (ng ml-1)
80
60
40
20
0
Estradiol (ng ml-1)
6.0
4.5
3.0
1.5
0
2005
2007
2008
2009
2010
2011
2005
2007
2008
2009
2010
2011

Fig 12


Testosterone
Estradiol
Hormone (ng ml-1)
50
40
30
20
10
0
b
y
b
z
Male
Female

Chapter 4: Detection of vitellogenin in shortnose sturgeon from Delaware River, USA

By Mark A. Matsche

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Summary

Expression of the female specific protein vitellogenin (VTG) is widely used as a sensitive indicator of endocrine disruption in fish, particularly in males. Analysis of blood samples for VTG was limited to 12 shortnose sturgeon collected in 2009 using an electrophoretic technique. Concentration of VTG in shortnose sturgeon (9.6 mg L^{-1} in two F5 females and 2.7 mg L^{-1} in one F2 female) is high in comparison to other sturgeon species of comparable reproductive stage, indicating that this species has naturally elevated levels or that VTG expression was enhanced in these fish in response to exposure to endocrine disrupting chemicals. VTG was not detected in 8 males or in one F2 female. Additional samples have been banked and await analysis.

Introduction

Vitellogenin (VTG) is synthesized in the liver, released into the bloodstream in response to circulating estradiol, taken up by developing oocytes where it is converted to egg yolk proteins. Production of VTG can be induced in fish by elevated endogenous estrogens and by exposure to exogenous estrogens (Webb & Doroshov 2011). VTG induction has been documented in a multiple fish species exposed to a variety of municipal and industrial effluents (McMaster 2001) and are often a sensitive indicator of endocrine disruption (Kime *et al*. 1999). The goal of this study was to examine plasma from male shortnose sturgeon for evidence of VTG expression and determine VTG concentration by reproductive stage in female fish.

Methods

Blood was collected from twelve shortnose sturgeon captured in fall, 2009, placed in a collection tube containing lithium heparin and centrifuged for 5 min. A 0.5 ml aliquot of plasma from each fish was transferred to an aprotonin-treated tube, placed on dry ice for transport and stored in -80ºC. Aprotonin-treated samples were shipped to Virginia Institute of Marine Science for analysis (Dr. Van Veld).

Plasma was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in discontinuous, pre-electrophoresed, 8% polyacrylamide gels in a Mini-Protean II apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Gels were stained with Pro-Q Diamond phosphoprotein gel stain (Molecular Probes, Eugene, OR, USA) according to manufacturer's directions. Gels were fixed overnight in 50% methanol and 10% acetic acid. Gels were washed with ultrapure water and stained for 90 min (Pro-Q Diamond stain) and destained twice for a total of 2 h with destaining solution (Molecular Probes). Fluorescent images were collected and quantified with a Fluorchem SP Imaging System (Alpha Inotech, San Leandro, CA, USA) at 365 nm.

Results

Bands of high molecular weight protein (≈150 kDa) were detected in two F5 females (Fig. 1) and a F2 female. Proteins corresponding to VTG were not detected in 8 males (F2-F5) or in 1 F2 female. Concentration of VTG in F5 females was approximately 3.5x higher than in the F2 female (Fig. 2).

Discussion

Although no VTG was detected in males in this study, sample size was too small to draw conclusions. Additional samples were collected from shortnose sturgeon in 2009-2011but the analytical lab at Virginia Institute of Marine Science is unable to process them at this time due to a cut in laboratory staff positions. Samples are stored in -80ºC and will be analyzed at the expense of Maryland DNR once suitable analysis services are available.

Plasmatic concentration of vtg was 2.7 mg L^{-1} in previtellogenic stage females, increasing to 9.6 mg L^{-1} in mature, pre-ovulatory females in this study. These concentrations high compared to other sturgeon species. In cultured hybrid sturgeon (*Huso huso* ♀ x *Acipenser ruthenus* ♂), VTG was <0.2 mg L^{-1} in F2 females increasing to approximately 1 mg L^{-1} in F5 females (Amiri *et al*. 1996). In cultured white sturgeon *Acipenser transmontanus*, VTG was <0.01 mg L^{-1} in previtellogenic females increasing to approximately 5 mg L^{-1} in F5 females (Linares-Casanave *et al*. 2003). In Missouri River shovelnose sturgeon *Scaphirhynchus platorhynchus*, VTG was approximately 0.01 mg L^{-1} in F2 females increasing to approximately 0.2 mg L^{-1} in F5 females (Wildhaber *et al*. 2007). There are likely interspecific differences in VTG in comparable reproductive stages and differences between cultured and wild fish. However, elevated levels of VTG evident in 3 fish examined in this study may indicate increased VTG induction in response to exposure to endocrine disrupting chemicals.

References

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Wildhaber, M.L. *et al.* (2007) Physical and hormonal examination of Missouri River shovelnose sturgeon reproductive stage: a reference guide. *Journal of Applied Ichthyology,* 23**,** 382-401.

Figure captions

Figure 1. Phosphoprotein staining of vitellogenin (VTG) in plasma from shortnose sturgeon captured in the Delaware River, 2009. For each fish, lane 1 contains 20 µg protein and lane 2 contains 5 µg protein. Reproductive stage given in box over each pair of lanes.

Figure 2. Plasma vitellogenin (VTG) from three female shortnose sturgeon captured in the Delaware River on November 2nd, 2009. Error bars indicate standard deviation and reproductive stage of fish is given on each bar.

Fig. 1

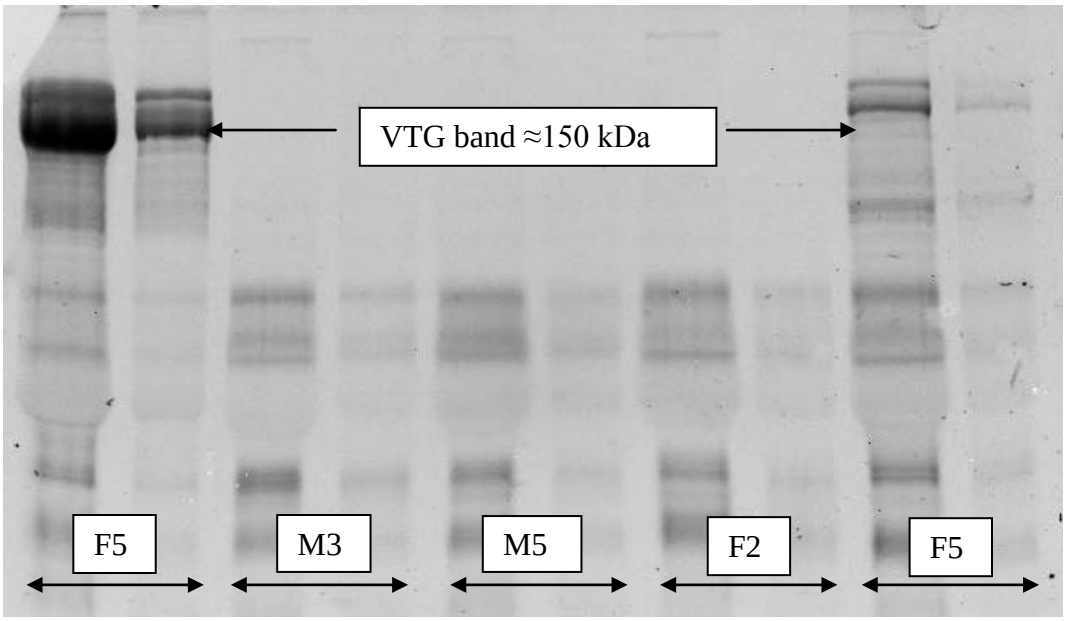
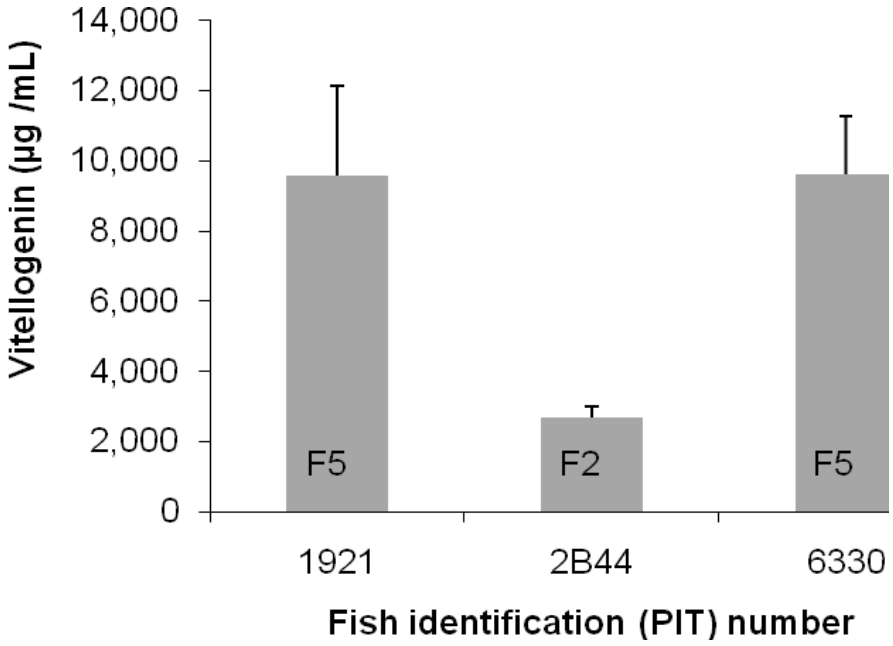


Fig. 2


14,000
12,000
10,000
8,000
6,000
4,000
2,000
0
Vitellogenin (µg /mL)
F5
F2
F5
1921
2B44
6330
Fish identification (PIT) number

Chapter 5: Attempts to detect estrogens in the Delaware and Cooper River

By Mark A. Matsche[1] and Jarrett Gibbons[2]

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Corresponding author: Mark Matsche, mmatsche@dnr.state.md.us

Summary

Effluents containing endocrine disrupting compounds, or EDCs, have been linked to altered development of gonadal tissue and hormone levels and changes in secondary sex-linked traits in fish and other aquatic animals. "Feminization" of fish, in which males express female characteristics including development of oocytes, is more commonly reported and is thought to be a consequence of exposure to estrogens or "estrogen-like" compounds. In this study, an ELISA assay for estrogen was used to detect presence of waterborne compounds with estrogenic activity in water collected from Delaware and Cooper Rivers. Water samples collected in either spring or fall, 2006-2011 from Delaware River, and in winter, 2005-2011 from Cooper River, South Carolina were negative for presence of estrogens. Future work should seek to investigate other sinks of estrogen accumulation in the environment such as in sediments. Development of toxicant assays to detect contaminants in tissue biopsies would alleviate the need for opportunistic sampling from dead specimens and may yield much insight into contaminant burdens in shortnose sturgeon.

Introduction

The endocrine system in fish and other animals consists of a series of glands and other tissues disseminated throughout the body that secrete hormones, which help guide development, growth, reproduction, behavior and other functions. Receptors in target organs detect presence of certain hormones, which when stimulated, can have a promoting or inhibitory effect. For example, timing and amount of estrogen and testosterone secreted in fish, along with other signals, stimulates changes in reproductive development. Treatment of fish with natural hormones can override genetic and other sex determinants, and has been used in research and in aquaculture to generate monosex populations for economic reasons. Inadvertent exposure to hormones released in municipal, agricultural or industrial effluents can also have a profound effect on the reproductive physiology of fish and other lower aquatic vertebrates. Natural and synthetic hormones are commonly identified in aquatic habitats. Also, numerous aquatic

pollutants have been identified as “endocrine disrupting chemicals” (EDCs), that is, they have the ability to interfere with natural hormone functions. A variety of effects have been attributed to exposure to EDCs including loss of secondary sex characteristics, changes in hormone levels, reduced egg fertility, reduced gamete production, feminization of males, masculinization of females, induction of female-specific proteins in males, developmental deformities, compromised immune functions and increased mortalities.

The goal of this study was to determine if estrogens or estrogen-like compounds could be detected in water collected from the Delaware and Cooper Rivers, indicating a potential waterborne source of exposure of EDCs for resident shortnose sturgeon.

Methods

Duplicate 1-L water samples were collected at or near sturgeon capture sites approximately 0.5 m below the river surface or approximately 1 m above the river substrate using an acrylic water sampler (LM1077, LaMotte Company, Chestertown, MD, USA). Water samples were placed on dry ice for transport and stored at -80ºC for up to 6 months.

Water samples were analyzed for estrone, estradiol and estriol using a commercial competitive ELISA test kit (E1/E2/E3 ELISA Kit, Japan Envirochemicals, Ltd, Tokyo, Japan) following manufacturer’s directions. Water samples were thawed, passed through a 1 µM glass fiber filter and split into 2 subsamples. One subsample of each sample was spiked with 1 or 10 ng ml^{-1} estradiol. Each sample was concentrated by passing the filtrate (spiked or unspiked) through a C18 cartridge (preconditioned with methanol and distilled water). The C18 cartridge was washed with 5ml distilled water, dried, and washed with 5 ml hexane. The analyte was eluted with 5 ml dichloromethane in a glass tube and then evaporated with nitrogen gas. The dried sample was dissolved in 2 ml methanol, passed through an aminopropyl cartridge (preconditioned with methanol) and collected in a glass tube. Remaining sample adhered to the aminopropyl cartridge was eluted with 5 ml methanol, added to the filtrate and solvent was evaporated with nitrogen gas. The dried material was dissolved in 5 ml methanol and adjusted with to 10% (v/v) with distilled water.

Spiked and unspiked samples and standards of known concentration were incubated in triplicate for 60 minutes at 22ºC. The microplate was washed 3 times using proprietary wash solution and incubated in color development solution for 30 min. Color development was halted by addition of stop solution and estrogen concentrations were

analyzed using a UQuant spectrophotometer (Biotek, Inc., Winooski, VT, USA) at a wavelength of 450nm. Cross reactivity with other hormones is reportedly <0.03% and coefficient of variation measured in this study was <8%.

Results

From 2006-2011 a total of 36 water samples were collected from the Delaware River and a total of 18 water samples were collected from the Cooper River. No estrogens were detected in any of the samples using the E1/E2/E3 ELISA assay. Analysis of spiked samples indicated that sample extraction and concentration was 87% efficient overall.

Discussion

Endocrine disrupting compounds include a wide range of chemicals, such as estrogens, progestogens, phytoestrogens and other organic compounds. Natural and synthetic estrogens have been identified as the greatest contributors to EDC effects observed in aquatic environments, often in physiologically active concentrations (ng L^{-1} range) (Metzler 2001). Development of sensitive, rapid-screening assays facilitates low-cost analysis of water samples for estrogens (Farré 2006).

The estrogens estrone and estriol were most commonly detected hormones in Delaware River watershed samples (Velicu & Suri 2009). Estradiols can be oxidized to estrone in waste water treatment processes and by commonly found algae in surface waters (Hanselman *et al*. 2003, Lai *et al*. 2001). Estriol is one of 3 major estrogens produced by humans and other animals, and sewage effluents and land applied manure may be significant sources of estriol (Velicu & Suri 2009). Hormones were more frequently detected in Delaware watershed sites characterized as suburban, although estradiols, estriol and estrone were detected ≥60% of agricultural sites potentially indicating multiple estrogen inputs to the Delaware watershed (Velicu & Suri 2009). Estrogen concentrations ranged from approximately 1 ng ml^{-1} (estradiols) to 6 ng ml^{-1} (estriol) (Velicu & Suri 2009), which were within the detection limits of the assay employed in this study. It is possible that estrogens were present in water sampled for this study but in undetectable levels or that estrogens deteriorated in samples during handling and storage.

References

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